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DNA condensation by copper(II) complexes and their anti-proliferative effect on cancerous and normal fibroblast cells

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ABSTRACT

In our search towards copper(II) based anticancer compounds, copper(II) complexes $[Cu(bitpy)_2](ClO_4)_2$ **1**, $[Cu(bitpy)(phen)](NO_3)_2$ **2** and $[Cu(bitpy)(NO_3)](NO_3)$ **3** were synthesized and characterized. All the three complexes contain the tridentate ligand bitpy, which bears biologically relevant benzimidazolyl head group, as one of the ligands. Because of the presence of the planar benzimidazolyl group in the bitpy ligand, the complexes exhibited intercalative mode of binding with DNA. The DNA binding constant, K_b, for complexes **1**, **2** and **3** were determined to be $(1.84 \pm 0.32) \times 10^4$, $(1.83 \pm 0.57) \times 10^4$ and $(1.87 \pm 0.21) \times 10^4\ M^{-1}$ respectively. All the three complexes possessed DNA condensing ability. The DNA condensing ability of the complexes was in the order **2** > **1** > **3**. The DNA condensation induced by these three complexes was found to be reversed in the presence of 1 M NaCl. In vitro cytotoxicity of three complexes was tested against osteosarcoma MG63 cell line as well as normal fibroblast NIH3T3 cell line by MTT reduction assay. Complexes **1** and **2** were found to be highly toxic towards MG63 than NIH3T3 cell line and both these complexes brought about cell death in the MG-63 cell line due to apoptosis. Whereas, complex **3** exhibited almost equal toxic effect towards both MG63 and NIH3T3 cell lines. Based on the fact that both complexes **1** and **2** brought about reversible condensation of DNA and induced apoptosis in osteosarcoma MG-63 cell line, it is hypothesized that they might possess potential pharmaceutical applications.

1. Introduction

Coordination complexes of transition metal ions occupy an important position in medicinal biochemistry. Platinum(II) based complexes, especially cisplatin and related compounds have fascinated inorganic chemists for a long time because of their anticancerous properties [1–3]. Cisplatin and its related compounds are widely used in chemotherapy. However, one of the significant problems with cisplatin and other platinum based drugs is their chemo-resistance. To surmount this issue, numerous transition metal complexes have been synthesized and tested for their anticancer activity [4–10]. In the past, copper(II) complexes have attracted special attention because of the fact that copper is an essential trace element and is required for normal cellular activity as a cofactor for many enzymes [11–13]. Some of the earliest compounds of copper(II) to gain interest of medicinal chemists due to their anticancer property are Cu(II) complexes of thiosemicarbazones and its derivatives [14–16]. Current focus of research on copper(II) complexes stems from their multivariate use; for example copper(II)–bipyridyl and copper(II)–phenanthroline complexes have been reported to exhibit antimicrobial, antiviral, anti-inflammatory and antitumor properties [17–20]. Moreover, copper(II), nickel(II) and ruthenium(III) complexes have also been demonstrated to function as enzyme inhibitors, DNA condensing agents, DNA cross-linking agents and chemical nucleases [17,21–24]. The properties of the copper(II) complexes are largely determined by the nature of ligands and donor atoms bound to the metal ion. Currently, for biological applications, ligands for copper(II) complexes are designed in such a way that they increase the lipophilicity of the complex for easy transport through cell membrane and also facilitate their binding to DNA and proteins. A number of copper(II) and copper(III) complexes containing biologically active ligands have been reported to have anti-proliferative, anti-cancerous, anti-bacterial, nuclease mimetic and SOD mimetic properties [11,25–31]. Current bioinorganic research is focused on improving the therapeutic properties of such

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complexes using bi-nuclear copper complexes as well as rationally designed mixed ligands to generate complexes with multiple functions. Recently, mixed ligand acetylacetone/quinoxaline complexes have been reported to exhibit nuclease and apoptosis-inducing activity [32–34]. Since 1999, our group has made significant efforts for the synthesis of ligands and respective metal complexes, which possess nuclease/protease activities as well as significant anti-proliferative effects [35–42]. It has been concluded from our previous studies that the cytotoxic effect of the metal complexes depends on their binding ability towards DNA. Even though a large number of copper(II) complexes have been shown to exhibit anticancer activity, only a few complexes have been reported to possess DNA condensation property. The complex $[Co(NH_3)_6]^{3+}$ has been shown to bring about DNA condensation [21]. The DNA condensing ability of this complex has been attributed to its electrostatic charge. DNA condensation ability is a prerequisite for gene therapy [43–46]. The challenge of successful gene therapy relies greatly on the development of effective and safe carrier, which is capable of compacting and delivering DNA. The DNA condensing ability is also essential for DNA transcription and replication. A molecule having both anticancer activity as well as gene targeting ability is expected to have significant application in medicinal chemistry.

In this manuscript, we describe the synthesis and characterization of three copper(II) complexes containing tridentate, bidentate and monodentate ligands. Benzimidazole (bzim) based ligand was chosen as a common ligand for all the three complexes because bzim has pharmaceutical and therapeutic applications [47]. Benzimidazole when incorporated into 4′ position of terpyridine ligand as benzimidiazolylterpyridine (bitpy), is expected to enhance the DNA binding ability due to its H-bonding ability. The efficacy of these complexes to bring about DNA condensation as well as their anti-proliferative effects on normal and cancerous fibroblast cell lines has also been examined.

2. Materials and methods

2.1. Materials

The chemicals, 2-acetyl pyridine, 1,10-phenanthroline, agarose, ethidium bromide, calf thymus DNA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and propidium iodide were purchased from Sigma Chemical Company (St. Louis, MO, USA). Trypsin, DMSO, Tris (hydroxymethyl) methylamine and Tris-borate-EDTA were purchased from Sisco Research Laboratory (Mumbai, India). Human osteosarcoma fibroblast cell line, MG-63 and mouse embryonic fibroblast noncancerous cell line NIH-3T3 were obtained from National Centre for Cell Sciences (NCCS, Pune, India). Caspase 3 and caspase 9 were purchased from M/s R & D systems (Bangalore, India). Caspase 8 was purchased from M/s Invitrogen. Minimum Essential Medium Eagle (MEM) was purchased from Hi Media Laboratories (Bangalore, India) and fetal bovine serum (FBS) was purchased from Cistron laboratories (Hyderabad, India). Milli-Q triply deionized water was employed for all the studies. The ligand bitpy was synthesized according to the reported literature [48]. ^{1}H- and ^{13}C NMR of bitpy is given in the supplementary information (Fig. S1).

2.2. Synthesis of complex, $[Cu(bitpy)_2](ClO_4)_2\cdot 2H_2O$ (**1**)

A methanolic solution (50 mL) of $Cu(ClO_4)_2\cdot 6H_2O$ (0.18 g, 0.5 mmol) and bitpy (0.35 g, 1 mmol) was refluxed for 30 min. A green solid that separated out upon slow evaporation of the solvent was filtered, and washed with diethyl ether and dried in vacuum. The complex was recrystallized from acetonitrile-water mixture. Yield: 79%. Found: C, 52.92; H, 3.31; N, 14.13%. Anal Calcd for $C_{44}H_{34}Cl_2CuN_{10}O_{10}$: C, 52.99; H, 3.44; N, 14.05%.

2.3. Synthesis of complex, $[Cu(bitpy)(phen)](NO_3)_2\cdot 3H_2O$ (**2**)

A methanolic solution (50 mL) of $Cu(NO_3)_2\cdot 3H_2O$ (0.12 g, 0.5 mmol) and bitpy (0.15 g, 0.5 mmol) was stirred at room temperature for 30 min. Subsequently, 1,10-phen (0.12 g, 0.5 mmol) was added to the above solution and stirred continuously for another 30 min. The resulting solution was reduced under pressure to yield dark green solid. The compound was recrystallized from acetonitrile- water mixture. Yield: 72%. Found: C, 52.86; H, 3.81; N, 16.27%. Anal Calcd: for $C_{34}H_{29}CuN_9O_9$: C, 52.95; H, 3,79; N, 16.35%.

2.4. Synthesis of complex, $[Cu(bitpy)(NO_3)]NO_3\cdot H_2O$ (**3**)

The complex **3** was synthesized by following the procedure described for the synthesis of complex **1** employing bitpy (0.35 g, 1 mmol) and $Cu(NO_3)_2\cdot 3H_2O$ (0.24 g, 1 mmol). The green colored precipitate obtained was recrystallized from acetonitrile-water mixture. Yield: 72%. Found: C, 47.58; H, 3.13; N, 17.71%. Anal Calcd: for $C_{22}H_{17}CuN_7O_7$: C, 47.61; H, 3.09; N, 17.67%.

2.5. DNA binding experiments

Stock solutions of metal complexes (10 mM) were prepared by dissolving the complexes in acetonitrile (500 μL) and making up to a total volume of 5 mL using 10 mM Tris buffer (pH 7.2). Absorption spectral titration experiments were carried out for the complexes **1**, **2** and **3** by maintaining a constant concentration of the complex (20 μM) and varying the CT-DNA concentration (5–120 μM). An equal amount of DNA was added to the cell in the reference compartment.

For viscosity measurements, the Ubberhold viscometer (1 mL capacity) was thermostated in a water bath maintained at 25 °C. The flow time for each sample was measured thrice using digital stopwatch and an average flow time was calculated. The flow rate for buffer (10 mM Tris), DNA (100 μM) and DNA with the copper(II) complexes at various concentrations (5–120 μM) was measured. The relative specific viscosity was calculated using the equation, $\eta = (t - t_o)/t_o$, where t_o is the flow time for the buffer and t is the observed flow time for DNA in the absence and presence of the complex. Data are presented as $(\eta/\eta_o)^{1/3}$ versus $1/R$ {R = [complex]/[DNA]}, where η is the viscosity of DNA in the presence of the complex and η_o is the viscosity of DNA alone [49,50].

Circular dichroic spectra were recorded with a Jasco J-815 spectropolarimeter at 25 °C using 0.1 cm path quartz cell. The concentration of CT-DNA (100 mM) was kept constant and the concentration of complexes **1**, **2** and **3** varied from 5 to 120 μM. The spectra were recorded in the spectral region of 220–300 nm.

Electronic spectra were recorded using a Perkin–Elmer Lambda 35 double beam spectrophotometer. Electrospray ionization mass spectra (ESI-MS) were obtained from Thermo Finnigan LCQ 6000 advantage max ion trap mass spectrometer using acetonitrile as carrier solvent. A stock solution of DNA was prepared by stirring DNA sample dissolved in 10 mM Tris HCl buffer (pH 7.2) at 4 °C and used within 4 days of preparation. The solution was exhaustively dialyzed against Tris buffer for 48 h and filtered using a membrane filter obtained from Sartorius (0.45 μM). The filtered DNA solution in the buffer gave a UV absorbance ratio (A_{260}/A_{280}) of about 1.9, indicating that the DNA was sufficiently free from proteins [51]. The concentration of DNA was determined using an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm [52]. All further experiments were carried out employing the prepared DNA solution in Tris buffer at pH 7.2.