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# Synthesis, structure, interaction with DNA and cytotoxicity of a luminescent copper(II) complex with a hydrazone ligand

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ABSTRACT

A luminescent copper(II) complex $[Cu^{II}(L)H_2O]_2SO_4$,**1a** was synthesized with the acyclic tridentate 2-hydroxy naphthaldehyde-2-pyridylhydrazone ligand, HL, **1**. The molecular structure of **1a** clearly shows that it is a monomeric copper(II) complex with $CuN_2OO_W$ coordination with slightly distorted square planar geometry. The asymmetric unit of **1a** contains two independent complex molecules and a sulfate anion. DNA binding interactions of **1a** have been investigated by absorption, emission, viscosity and thermal denaturation studies. The cytotoxic activity of **1a** was measured in vitro against the HeLa cells. The $LD_{50}$ value was calculated as 4.97 μM. There was no nuclear fragmentation observed after treatment with **1a**, which revealed that the copper(II) complex **1a** was not capable of inducing apoptosis. The cell cycle analysis of **1a** indicated a dose dependent decrease of $G_0/G_1$ and S-phase cell population and dose-dependent increase of $G_2$/M population compared with untreated control cell.



## 1. Introduction

Studies on the interaction of small molecules with DNA continue to attract considerable attention due to their importance in cancer therapy and molecular biology [1–6]. In this respect, transition metal complexes that are capable of binding DNA under physiological conditions are of interest in the development of metal-based anticancer agents [7–11]. The use of such complexes in footprinting studies, sequence specific DNA binding agents, diagnostic agents in medicinal applications and for genomic research has generated current interests to develop this chemistry further [12–17].

Copper is an essential transition metal ion in the active sites of many enzymes present in the human body and plays an important role in various physiological processes [18–22]. Due to its bioessential activity and oxidative nature, suitably designed copper(II) complexes are of great interest in the development of tumor therapeutics, antibacterial and antimicrobial agents [23–26].

Copper(II) complexes containing heterocyclic bases have been extensively explored in virtue of their strong interactions with DNA and cytotoxic activity [27–30]. Design of metal-based pharmaceuticals depends on the ligand framework, the choice of metal ion, and its oxidation state [31,32]. Previously, we have reported a family of copper(II) complexes with hydrazone ligands containing the –N=C–CH=NH–C=N– motif, which is a potential tool for development of multidentate chelators with functional diversities [33–37]. Moreover, modulation of ligand frame by proper choice of donor atoms significantly influences the structural and functional behavior of the complexes [38–40]. Herein, we have synthesized the acyclic tridentate 2-hydroxy naphthaldehyde-2-pyridylhydrazone ligand HL, **1**, with conjugated aromatic core and its copper(II) complex, **1a** in order to increase the versatility of the hydrazone system. Interestingly the molecular structure of **1a** shows that each asymmetric unit contains two $CuN_2OO_W$ molecules with sulfate as an anion. It shows DNA interaction and cytotoxic activity with HeLa cells as revealed from cell viability, cell morphology.

## 2. Experimental

### 2.1. Materials

All starting materials and solvents were purchased from Sigma Aldrich Chemical Company and used without further purification unless otherwise stated. Ethidium bromide and DNA (CT-DNA) were purchased from Bangalore GeNei. Propidium iodide, Hoechst dye and RNase A were purchased from Sigma Chemicals (USA). Culture media MEM and serum was purchased from HiMedia, India. Other molecular biology grade fine chemicals were procured locally.

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### 2.2. Physical measurements

A Perkin Elmer 2400 C Elemental Analyzer was used to collect microanalytical data (C,H,N). Sartorius CP64 balance was used for weighing purpose. FTIR data were collected with the help of FTIR Perkin Elmer L 120-000A. UV–Vis spectra were recorded on Shimadzu UV-1700 spectrophotometer and corrected for background due to solvent absorption. Emission spectra were carried out with the help of Perkin Elmer LS 50B Luminescence Spectrometer. For binding constant measurements solutions were prepared in methanol at a fixed concentration of HL, **1** ($1.0 \times 10^{-5}$ M) and at a concentration of metal ions ranging from (1.0–10.0) $\times 10^{-6}$ M at room temperature. FACS Calibur™ (BD Bio-science, USA) fluorescence-activated cell sorter was used for cell cycle analysis. Axioskop 2 plus (Carl Zeiss) fluorescence microscope was used to study cell morphology and apoptosis.

### 2.3. Ligand and complex

#### 2.3.1. Synthesis of HL, *1*

The ligand HL, **1,** was prepared by the 1:1 condensation of 2-hydrazinopyridine (0.021 g, 0.20 mmol) and 2-hydroxy naphthaldehyde (0.036 g, 0.20 mmol) in methanol. On recrystallization from methanol, pale yellow crystals separated out which was dried over fused $CaCl_2$. Yield: 0.044 g (82%). $C_{16}H_{13}N_3O$, **1a**: C, 73.00; H, 4.94; N; 15.96. Found: C, 73.04; H, 4.96; N, 15.99%. FTIR (KBr disc): $\nu_{-OH}$, $\nu_{-NH}$ (3106, 3009), $\nu_{-CH=N}$ (1596), 1459, 1379, 1277, 1237, 1185, 1141, 1029, 988, 952, 899, 848, 814, 767, 738, 671, 648, 636, 620 $cm^{-1}$.

#### 2.3.2. Synthesis of $[Cu(L)H_2O]_2SO_4$, *1a*

To a methanolic solution of copper(II) sulfate pentahydrate (0.20 mmol, 0.07 g) was added a solution of HL, **1**, (0.20 mmol, 0.04 g) in methanol (5 ml) and the resulting green solution was stirred for 0.5 h. The solvent was evaporated by rotary evaporator and the solid obtained was recrystallized from acetonitrile. Yield: 0.08 g (84%). *Anal.* Calc. for $C_{32}H_{29}Cu_2N_6O_8S$, **1a**: C, 48.97; H, 3.69; N; 10.71. Found: C, 48.95; H, 3.67; N, 10.69%. Found: C, 26.58; H, 2.82; N, 11.27%. FTIR (KBr disc): 3091($\nu_{-NH}$), 1542($\nu_{-CH=N}$), 1483, 1460, 1388, 1361, 1340, 1290, 1261, 1247, 1193, 1145, 1112, 1043, 967, 921, 880, 870, 855, 824, 785, 759, 703, 645 $cm^{-1}$.

### 2.4. DNA binding experiments

The DNA binding experiments were performed at 25.0 ± 0.2 °C. The concentration of CT-DNA was determined from its absorption intensity using the known molar extinction coefficient value of 6600 $M^{-1}$ $cm^{-1}$ at 260 nm [41]. The UV–Vis titration of **1a** was performed in buffer (50 mM NaCl–50 mM Tris–HCl, pH 7.2) medium using a fixed complex concentration to which increments of the DNA stock solution ($0.0 \times 10^{-5}$–$4.0 \times 10^{-5}$ M) was added. The resulting solution was incubated for 10 min before absorption spectra were recorded.

During the fluorescence quenching experiment, the DNA was pretreated with ethidium bromide (EB) for 0.5 h and the complex **1a** was added to this mixture. On excitation at 490 nm, emission peak was observed between 500 and 700 nm.

DNA-melting experiments were carried out by monitoring the absorbance of CT-DNA (100 μM) at 260 nm with varying temperature in the absence and presence of complex **1a**, in a 2:1 ratio of DNA to complex with a ramp rate of 0.5 °C $min^{-1}$ in Tris buffer (pH 7.2) using a peltier system attached to the UV –Vis spectrophotometer.

For viscosity measurements, the Ubbelohde viscometer was thermostated in water-bath maintained at 25 °C. The flow time for each sample was measured three times using digital stopwatch and an average flow time was calculated. The rate of flow of the Tris buffer (pH 7.2), DNA (100 μM) and DNA with the complex, **1a** at various concentrations were measured. The relative specific viscosity was calculated using the equation $\eta = (t - t_0)/t_0$, where $t_0$ is the flow time for the buffer and $t$ is the observed flow time for DNA in the absence and presence of the complex **1a**. Data are presented as $(\eta/\eta_0)^{1/3}$ versus $1/R$ (where $R$ = [complex]/[DNA]), $\eta$ is the viscosity of DNA in the presence of the complex and $\eta_0$ is the viscosity of DNA alone [42,43].

### 2.5. Cell culture

Human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Sciences, Pune, India. HeLa cells were grown in MEM supplemented with 10% bovine serum (complete medium) at 37 °C in humidified atmosphere containing 5% $CO_2$.

#### 2.5.1. Cell viability and morphology

After treatment with different concentrations of the complex **1a** (0–10 μM) for 17 h the HeLa cells were harvested, washed with phosphate buffered saline (PBS) twice. Then the cells were incubated with 0.2% trypan blue for 5 min at room temperature and counted by hemocytometer under light microscope. Each experiment was repeated four times and the mean of % viable cells at each dose was compared with the mean of untreated control using one way ANOVA with a post hoc test viz. Dunnett's test. The *p*-values were denoted in the figures.

Cell morphology study was performed according to our previous procedure with slight modification [37]. In brief, cells grown over cover slips for overnight, was treated with different concentrations of **1a** (0–10 μM) for 17 h. The cells over cover slips were washed with phosphate buffered saline (PBS) twice and placed over clean microscopic slides. The photographs were taken using Axio vision 3 software package (Carl Zeiss).

#### 2.5.2. Detection of apoptosis by nuclear fragmentation

Detection of nuclear fragmentation was done as reported earlier [44] with some modification. Briefly, HeLa cells were grown over cover slips (placed inside the culture plates) overnight and then treated with different concentrations of **1a** (0–10 μM) for 17 h. Now the cover slips were washed twice with PBS buffer and the cells were fixed using 2 ml of fixative solution of methanol: acetone (1:1) for 1 h at 4 °C. Again the cells were washed twice with PBS buffer. Finally it was stained with Hoechst dye (1 μM), incubated for 10 min in dark, and washed with PBS. Cells were examined by fluorescence microscope (Carl Zeiss) using the appropriate filter. Apoptotic cells were distinguished by nuclear fragmentation and chromatin condensation. The apoptotic cells as well as the normal cells were randomly counted and percentage of apoptotic cells was calculated at each dose. The mean percentage of apoptotic cells with standard deviation was calculated from four independent experiments.

#### 2.5.3. Cell cycle analysis by flow cytometer

The cells were prepared for FACS analysis as our earlier studies [37,45]. In brief, after 17 h treatment, the cells were trypsinized, washed twice with cold phosphate buffered saline (PBS), fixed with 70% chilled ethanol in PBS for 5 h at 4 °C, and then stained with a solution containing 10 μg/ml propidium iodide (PI), 100 μg/ml of DNase-free RNase A (Sigma), and 0.1% (v/v) Triton X-100 in the dark for 30 min at room temperature. Cell cycle analysis was done by a FACS Calibur (BD Bioscience, USA) [46] at 488 nm excitation. 20,000 cells were taken for each set and fluorescence data were plotted using CELLQUEST software (Becton Dickinson). Using this raw data, the percentages of cells in each phase of the cell cycle was obtained using ModFit LT software. Three independent