Research Paper

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The Cytotoxic Activity of the New Unsymmetrical and Symmetrical "End-Off" Copper Complexes in A549 Cell Line

Chemistry

KEYWORDS : Schiff base, metal complexes, copper, cytotoxicity, cancer cell line

S.H. Anjana Lakshmi	University of Madras, Guindy Campus, Tamil Nadu, India. PIN –600025
M. Kandaswamy	University of Madras, Guindy Campus, Tamil Nadu, India. PIN –600025
T. N. Sathya	International Institute of Biotechnology and Toxicology, Kanchipuram district, Padappai, Tamil Nadu. PIN – 601301.

ABSTRACT

We synthesized four copper complexes of Schiff Base. Compounds 1 and 2 are unsymmetrical binuclear complexes while compounds 3 and 4 are symmetrical binuclear complexes. The structure of the compounds was determined and the in vitro cytotoxic activity of the complexes was assessed on A549 lung carcinoma cell line. The metal complexes were dissolved in water (compound 2 and 4) or DMSO (compound 1 and 3). Various concentrations (1000, 500, 250, 100, 50, 25, 10, 5 and 2.5 μM) of each compound were prepared for exposure to A549 cell line. MTT - Cell viability assay was performed to assess the cytotoxicity of the compounds. Out of the four copper complexes synthesized in this study, compound 4 exhibited notable cytotoxic effects in human lung (A549) cancer cell lines after 24-h treatment. Further investigations on the precise mechanism of action are underway to achieve a better understanding over the cytotoxicity mechanism.

1. Introduction

The science of bioinorganic chemistry involving the synthesis and biological investigation of inorganic complexes is gaining impetus [1-5] . In this regard, Schiff bases and their complexes have been studied for their interesting and important properties such as their ability to bind reversibly to oxygen, catalytic property, amino group transfer, photochromicity and complex formation. The high affinity in chelating with transition metal ions has encouraged the preparation of solid complexes [6]. Recent advances in inorganic Chemistry have instigated the synthesis of Schiff base metal complexes with organic ligand of interest, which can be used as therapeutic agent. Some Schiff bases and their metal complexes containing copper, Nickel, Zinc and Cobalt have been reported to possess antitumor activity [7-8] proposed that, transition metal complexes of Schiff base ligands have the potential to be the future medicinal candidate provided they are properly designed.

One such transition metal that has been of interest to the scientific community is Copper. Copper is an important trace element involved in redox reactions and is found in all living organisms. It is a vital component of various enzymes. Copper compounds have been investigated in detail owing to the assumption that endogenous metals may be less toxic. Further, Copper complexes are being extensively studied for their application as antibacterial, antifungal, anti-inflammatory antitumor agents [9].

With this background, in the present study, we synthesized four copper complexes of Schiff Base. Compounds 1 and 2 are unsymmetrical binuclear complexes while compounds 3 and 4 are symmetrical binuclear complexes. The structure of the compounds was determined and the *in vitro* cytotoxic activity of the complexes was assessed on A549 lung carcinoma cell line.

2. Materials and Methods

2.1.

2.2. Synthesis of the unsymmetrical binuclear complexes 1, 2

The precursor compound, 2, 6-diformyl-4-methyl phenol was prepared by the literature methods [10]. Initially, the mononuclear complex was prepared by, 2, 6-diformyl-4-methyl phenol (3.0 g ; 18 mmol) in warm Dimethylformamide (30ml) and 1,4-diaminocyclohexane (1.0 g ; 0.9 mmol) was added drop wise under stirring. The Cu $(OAc)_2.2H_2O$ 1.8g; 0.9mmol respectively) was added and the solution was stirred at 60°C for 1h. The resulting mononuclear complex was precipitated. The solid was separated by filtration and washed with 2-propanol and diethyl ether. Further, a methanolic solution of Copper (II) per chlorate hexahydrate (0.9mmol) was added dropwise to the previously prepared mononuclear complex and was also treated with different amines, 1,8- naphthyl diamino(0.9 mmol), Orthophenylenediamine(0.9 mmol), Aniline (1.8mmol) and Benzyl amine(1.8mmol) respectively was dissolved in 20ml of Acetonitrile added drop-wise to the reaction mixture. The solution was refluxed for 12 hrs. Each amine corresponds to form the desired unique complexes 1, 2 respectively. The resulting solution was then filtered in hot condition and allowed for slow evaporation at room temperature, washed with methanol and dried in vacuum. The corresponding product was recrystallized with acetonitrile/dry ethanol.

2.3.

2.4. Synthesis of the symmetrical binuclear complexes compound (3) and (4):

An ethanolic solution of Copper perchlorate hexahydrate was added dropwise to the 2,6-diformyl-4methylphenol in 20ml of ethanol to that diethylenetriamine is added very slowly dropwise in the ratio of 2:1:2 respectively and later triethyleneamine was added. [11] If any precipitate occurs, supposed to filter it immediately after the addition of triethyleneamine. The reaction mixture was allowed to stir at Ice cold condition for 6 h. The resulting solution was then filtered immediately and allowed for slow evaporation at room temperature, washed with methanol and dried in vacuum. The corresponding product was re-crystallized with ethanol. Further, to an ethanolic solution of the prepared binuclear complex, the corresponding Aldehydes- viz., benzaldehyde and naphthaldehyde was added slowly drop wise to the reaction mixture in the ratio of 1:2 respectively. The reaction mixture was allowed to stir at room temperature condition for 5h and it is refluxed at 60°C for 1h.The resulting solution was then filtered in hot condition immediately and allowed for slow evaporation at room temperature. The obtained product was washed with methanol and dried in vacuum. The corresponding product was re-crystallized with acetonitrile and chloroform mixture.

2.5. Characterizations of the Compounds 1-4

2.3.1. Infrared spectral analysis

The IR spectra were recorded on Perkin Elmer FTIR, spectrometer with samples prepared as KBr Pellets

2.3.2. Elemental analysis

The C, H, N contents of the ligands and

complexes were carried out using a Carlo Erba Elemental analyzer Model 1106.

2.3.3. ESI-Mass spectral Analysis

Electrospray ionization mass spectral measurements were done using Thermo Finnigan LCQ-6000 Advantages Max-ESI mass spectrometer.

2.3.4. Electrochemical studies

Electrochemical measurements were performed at room temperature. The cyclic voltammograms of 10^{-3} M solution of complexes were obtained on a CHI600A electrochemical analyzer. The measurements were carried out under oxygen free condition using a three electrode cell in which glassy carbon electrode was the working electrode, saturated Ag/AgCl electrode was the reference and platinum wire was used as the auxiliary electrode. Tetra(n-butyl) ammonium perchlorate (TBAP) was used as supporting electrolyte and the concentration of TBAP was 0.1 M.

2.4.

2.5. Cell line – source and maintenance

The A549 cell line was obtained from the American Type Culture Collection (ATCC) Rockville,MD, USA). Culture media, antibiotics, MTT were purchased from Sigma, USA.

2.6. Preparation of test concentrations

The metal complexes were dissolved in water (compound 2 and 4) or DMSO (compound 1 and 3). Various concentrations (1000, 500, 250, 100, 50, 25, 10, 5 and 2.5 μM) of each compound were prepared for exposure to A549 cell line.

***2.7. In vitro* cytotoxicity in cell line**

The *in vitro* cytotoxicity of the 4 copper complexes was assessed in A549 cell line (ATCC, USA). The cell line was maintained on Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% Fetal Calf Serum. A sub-confluent monolayer of the cell line was trypsinized and 1 x 10^5 cells were seeded in each well of a 96 well plate. One plate was used for each compound. Control wells were prepared by addition of culture medium without the compounds. The plates were incubated at 37°C in a 5% CO2 incubator for 24 h. The plates were incubated at 37ºC for 24 hours (the cells reach confluence in the plate during this time). At 24 hours, the cells were treated with various concentrations (1000, 500, 250, 100, 50, 25, 10, 5 and 2.5 μM) of the metal complexes and incubated for a period of 24 hours.

Upon completion of the incubation, MTT dye solution was added to each well to a final concentration of 5 mg/mL. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] cell viability assay was performed to assess the cytotoxicity of the compounds [12]. The MTT assay for cell injury is based on the ability of mitochondrial dehydrogenases of viable cells to reduce MTT to an insoluble purple formazan product which can be quantified spectrophotometrically. This formazan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity.

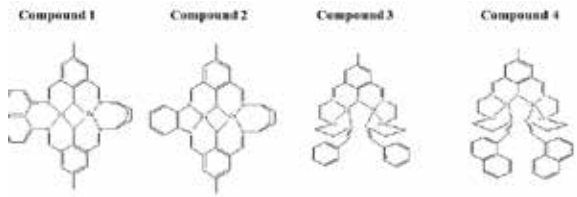
After 4 h incubation, the culture media were discarded and the wells were washed with phosphate buffer saline, followed by addition of DMSO and subsequent incubation for 30 min. The optical density of each well was then a Microplate Reader at a wavelength of 540 nm. The IC50 values were determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells remained viable relative to the control. Each experiment was repeated at least three times to obtain mean values.

3.

Results

3.1. Structure of the complexes

The structure of the copper complexes of Schiff base are presented below



3.2. Infrared spectral analysis

The IR spectra of the unsymmetrical and symmetrical macrocyclic binuclear copper(II) complexes (1 – 4) [13] have been carried out is depicted in figure s1-s4. The complexes shows characteristic peak around 3400 cm^{-1} due to stretching vibration of OH group. A sharp peak around 2925 cm^{-1} is assigned to the asymmetric stretching vibration of ν(C-H). The presence of a strong band around in the range of 1615 - 1635 cm^{-1} is assignable to the ν(C=N) vibration of binuclear Schiff base complexes. A strong band in the region at 1100 cm^{-1} and sharp band in the region at 626 cm^{-1} could be due to the antisymmetric stretch and antisymmetric bend of perchlorate ions. The perchlorate ions have not shown splitting peaks in the region around 1100 cm^{-1} due to the fact that the perchlorate ion absent in the complexes were uncoordinated to the Cu(II) ions. The important strong band observed around at 440 cm^{-1} for all the macrocyclic Cu(II) complexes are assignable to ν(M-O) vibrations.

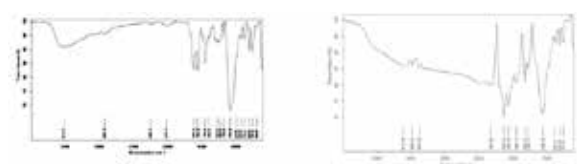


Figure s1 and s2 : IR spectra of the Compound 1 and 2 respectively (from left to right)

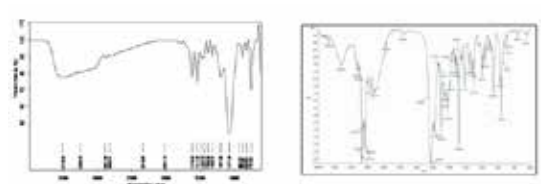


Figure s3: IR spectrum of compound 3

Figure s4: IR spectrum of compound 4

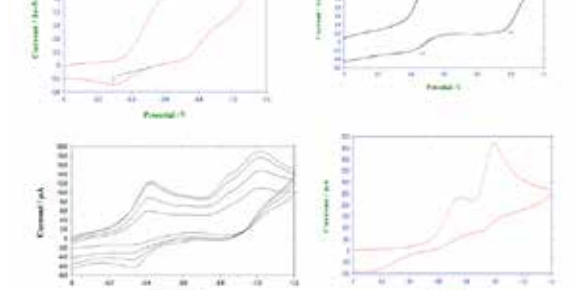


Figure s5-s8: Cyclic Voltammetric studies of the compound 1, 2, 3 and 4 respectively (clockwise)

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3.3. Elemental analysis

3.3.1. Compound 1

Elemental analysis (%) for $C_{30}H_{28}Cu_2N_4O_2$

Calculated : C= 59.69, H= 4.68,

Cu= 21.05, N= 9.28, O=5.30

Found : C= 58.25, H= 4.08,

Cu= 20.99, N= 9.08, O=4.96

3.3.2. Compound 2

Elemental analysis (%) for $C_{34}H_{30}Cu_2N_4O_2$

Calculated : C= 62.47, H= 4.63,

Cu= 19.44, N= 8.57, O=4.89

Found : C= 61.40, H= 4.08,

Cu= 18.79, N= 8.08, O=4.71

3.3.3. Compound 3

Elemental analysis (%) for $C_{31}H_{37}Cu_2N_6O$

Calculated : C= 58.47, H= 5.86,

Cu= 19.96, N=13.20, O=2.51

Found : C= 58.35 H= 5.69,

Cu= 19.85, N=13.17, O=2.29

3.3.4. Compound 4

Elemental analysis (%) for $C_{39}H_{41}Cu_2N_6O$

Calculated : C= 63.57, H= 5.61,

Cu= 17.25, N=11.40, O=2.17

Found : C= 63.44, H= 5.39,

Cu= 17.21, N=11.30, O=2.7

3.4. ESI Mass Spectral Analysis

The ESI mass spectra were carried out for the binuclear copper(II) complexes **(1-4).** The ESI mass spectrum of the binuclear complex 1-**4** shows the peak at m/z = 653 (l) , is in good agreement with the molecular weight, 603 (2), 638 (3), and 738 (4) corresponding to the [M+2] molecular ion peak. The **ESI** mass spectral data of the copper (II) complexes confirm the proposed structure of the complexes.

3.5. Electrochemical studies

The electrochemical behavior of unsymmetrical and symmetrical macrocyclic binuclear Cu(II) complexes 1 - 4 has been studied by cyclic voltammetry (CV) in CH_3CN containing 0.1 M TBAP is depicted in the figure s5–s8. The unsymmetrical Cu(II) complexes **1** – 4 are associated with two quasi-reversible reduction waves. The first reduction potential ranges from -0.25, to -0.70 V and the second redox potential lies in the ranges from -0.74 to -1.22 V. Its a single electron transfer process, and this can be assigned to the redox couple $Cu^{II}Cu^{II}$/ $Cu^{II}Cu^{I}$. The second quasi reversible wave can be attributed to the reduction of $Cu^{II}Cu^{I}$ in to $Cu^{I}Cu^{I}$ species. Based on these observations, it is reasonable to suggest that the reduction process may involve the stepwise redox processes. Two different reduction waves of all the Cu(II) complexes may be due to Cu(II) ions present in two different compartments as well as electrostatic effects arises during redox processes. When the first Cu(II) ion is getting reduced then the charge of the complex decreases from +2 to +1. Therefore, the second Cu(II) ion reduced at higher negative potentials. It was reported that the metal ion in imine [14] moiety reduces at less negative potential. It has been suggested [15-17] that reduction of electron density on the metal ions and distortion in geometry favors the reduction process at less negative potentials [18]. The larger electronegative oxygen present in phenolate moiety makes the copper(II) ion to reduce at more positive potentials than the Cu(II) ion, which is located in azomethine moiety [19]. As the size of macrocycle is increased, shifting of both first and second reduction potentials towards anodic is observed for the binuclear Cu(II) complexes. The ΔE values of first redox couple of Cu(II) complexes 1 to 4 in the range of 80 mV to 100 mV and second redox couple in the range of 90 to 130 mV at a scan rate of 50 mVs^{-1}. From the cyclic voltammetric studies it can be stated that the complexes containing aromatic diimines get reduced at higher negative potential than that of the complexes containing aliphatic diimine, The higher reduction potential can be attributed due to the greater planarity and electronic properties those are associated with aromatic rings

3.6. *In vitro* cytotoxicity in A549 cell line

The IC_{50} values of the 4 compounds were determined using the formula:

$$IC50 = \frac{\text{Mean OD of the control} - \text{Mean OD of the treated cells}}{\text{Mean OD of the control}} \times 100$$

Compound 1 exhibited an IC_{50} value of 404.7 µM, Compound 2 – 859.6 µM, Compound 3 – 148.9 µM and Compound 4 - **3.58 µM**. Considering the IC_{50} values, it is evident that Out of the four compounds tested, compound 4 is considered to be more effective in exhibiting cytotoxic effect on A549 lung carcinoma cells. Cytotoxic activity of copper complexes is generally attributed to the generation of Reactive Oxygen Species (ROS) by copper ions.

4. Conclusions .

Schiff bases are an interesting group of ligands that coordinate with metal ions via azomethine nitrogen. They have been studied extensively because of increasing recognition of their role in biological system. Schiff bases act as superior chelating agents for metals of transition and non-transition group and exhibit remarkable biological activities. Coordination of these compounds with metal ions, such as copper and nickel enhance their activities. Studies have shown that metal complexes of Schiff's base ligands have better antimicrobial and anticancer activities as compared to Schiff's bases [20]. Copper complexes have been reported to exhibit biological activities such as DNA damage, Single-strand breaks in supercoiled plasmid DNA in vitro, strong anti-proliferative activity, production of ROS, cell death by non-apoptotic mode, changes in redox potential, inhibition of cell cycle, etc., in various cell lines including A549 lung carcinoma cell line [21-24]. In the present study, we attempted to prepare, characterize and assess the biological (cytotoxic) activity on A549 lung carcinoma cell line. Out of the four complexes tested, the fourth compound exhibited appreciable cytotoxic activity on this cell line. In general cytotoxic acitivty of copper complexes in cancer cell lines (anticancer activity) is related to their ability to produce reactive oxygen species (ROS). Copper ions reduce hydrogen peroxide to hydroxyl radical. The effect of these ROS could be the primary cause of cytotoxicity in this cell line as outlined in the literature [9 and 25]. Another factor that could have contributed to the marked cytotoxic effect exhibited by compound 4 could be the increased aromaticity. Copper-based complexes have been reported to exhibit anti-neoplastic potency in various cell lines including human ovarian carcinoma (CH1), murine leukemia (L1210), and various cervico-uterine carcinomas. Such effects of copper complexes are on par or even exceeding the effects exhibited by Cisplatin[26-27]. The in vitro cytotoxicity of two copper-

2,2,-bipyridine complexes towards five selected tumor cell lines HepG-2, HeLa, NCI-H460, MCF-7, and HL-6 with IC50 value at a concentration of 50 μM were reported by the literature method [28] **Fei et al (2013)**.

In the present study, out of the four compounds tested, one of the compounds (Compound 4) exhibited an IC50 value at a concentration of 3.58 μM which is an appreciable cytotoxic effect and has the scope to be developed as a potential anticancer drug. However, detailed investigations on the mechanism of actionare-warranted. It is reported that Cu complexes of Schiff bases exhibit their antiproliferative activities owing to the properties of the metal center or the coordinated ligands; the structural and electronic properties of the coordinated ligands also dictate the effect [29-35] states that copper coordination compounds have remarkable application potential and an intensive research is required to successfully apply as anticancer drugs in future

In conclusion, our studies demonstrate that, out of the four copper complexes synthesized in this study, **compound 4** exhibited notable cytotoxic effects in human lung (A549) cancer cell lines after 24-h treatment. Further investigations on the precise mechanism of action are underway to achieve a better understanding over the cytotoxicity mechanism.

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