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Research paper

Pyridazine-based heteroleptic copper(II) complexes as potent anticancer drugs by inducing apoptosis and S-phase arrest in breast cancer cell

Ummer Muhammed Rafi[a], Dharmasivam Mahendiran[a], Venkat Gayathri Devi[b], Mukesh Doble[b], Aziz Kalilur Rahiman[a,*]

[a] Post-Graduate and Research Department of Chemistry, The New College (Autonomous), Chennai 600 014, India
[b] Bioengineering and Drug Design Laboratory, Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600 036, India

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ABSTRACT

A new series of heteroleptic copper(II) complexes of the type $[Cu(L^{1-3})(diimine)](ClO_4)$ (**1–6**) have been synthesized using three pyridazine-based ligands (3-chloro-6-(salicylidenehydrazinyl)pyridazine (HL^1), 3-chloro-6-(4-diethylaminosalicylidenehydrazinyl)pyridazine (HL^2) and 3-chloro-6-(5-bromosalicylidenehydrazinyl)pyridazine (HL^3), and diimine (2,2′-bipyridine (bpy) or 1,10-phenanthroline (phen)) as co-ligands. The ligands and their copper(II) complexes have been characterized by elemental analyses and spectroscopic methods. The copper(II) complexes display ligand-field band in the region 641–661 nm suggesting square pyramidal geometry. The optimized structures of the complexes and their molecular orbital calculations obtained by the density functional theory (DFT) also showed five coordinated distorted square pyramidal geometry around the copper (II) ion. The cyclic voltammetric analyses of copper(II) complexes exhibit one-electron irreversible reduction wave (E_{pc} = −0.596 to −0.641 V) in the cathodic potential region. Anti-proliferative activity of the complexes against breast cancer MDA-MB-231 cell line was evaluated by MTT cell proliferation assay, and the clonogenic assay revealed improved cytotoxicity for the complexes with potency higher than the standard drug cisplatin. Since the complexes **3** and **4** with diethylamino substituent displayed higher anti-proliferative activity than the other complexes, these complexes were chosen for apoptosis and cell cycle analysis. The apoptosis induction was analyzed by AO/EB staining, and the flow cytometry showed the inhibition of cell growth at the S-phase of the cell cycle. Additionally, the interaction of copper(II) complexes with FGFR kinase receptor have been studied by *in silico* molecular docking studies.

1. Introduction

Diazines are six-membered aromatics with two nitrogen atoms, which belong to the most important *N*-containing heterocycles. According to the relative position from the nitrogen atoms, three different structures can be distinguished *viz.*, pyridazine (1,2-diazine) [1], pyrimidine (1,3-diazine) [2] and pyrazine (1,4-diazine) [3]. Among these, pyridazine is a highly π-deficient aromatic compound and the most basic with pKa = 2.3, which favors protonation, hydrogen bond formation and chelation through nitrogen atoms of the pyridazine ring. The pyridazine core has also been found in a large range of biologically active structures such as herbicides [4] and pharmaceuticals [5,6]. The metal(II) complexes derived from pyridazine-based ligands exhibited anticancer activity against non-small-cell lung (A549), breast (MCF-7/MDA-MB-231), cervical (HeLa), hepatoma (HepG-2) and osteosarcoma (MG-63) cancer cell lines [7–9]. A series of compounds derived from imidazo[1,2-*b*]pyridazine scaffold was found to act as tumor necrosis factor (TNF-α) production inhibitor [10], and diarylpyridazine scaffold functionalized with different sulfenamide and sulfonamide acted as selective cannabinoid receptor (CB_1R) antagonists [11]. Pyridazine-bridged complexes also exhibit photo-physical properties favorable for luminescent bio-imaging applications [12].

Cancer remains the second leading cause of human deaths after cardiovascular diseases [13]. The design and synthesis of new metal-based anticancer agents with a better biological activity, better selectivity, lower toxicity and different mechanism of action to solve the unresolved clinical problems of cisplatin analogous drugs have been the focus of bioinorganic and medicinal chemists [14,15]. Many metal complexes have been extensively investigated as they act as potent growth inhibitors of human cancer cells by *in vitro* and *in vivo* experiments [16]. More efforts are being made to synthesize Ni(II), Cu(II), Zn(II) and Ru(II) complexes to act as better anticancer drugs, which could

* Corresponding author.
E-mail address: akrahmanjkr@thenewcollege.in (A.K. Rahiman).

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overcome the limited activity of cisplatin [17,18]. Among the metal(II) complexes, copper(II) complexes exhibit remarkable anticancer activity with lower toxicity than the platinum compounds, and act as potential reagents for DNA cleavage both oxidatively [19] and hydrolytically [20]. Copper(II) complexes derived from 1-(6-chloropyridazin-3-yl)-5-(2-hydroxyphenyl)-3-methyl-1H-pyrazole-4-carboxylic acid methyl ester, which exhibited cytotoxicity activity against leukemia HL-60 and NALM-6 cell lines [21], and pyridazolato-bridged copper(II) complexes, which exhibited anti-proliferative and apoptosis inducing activity against estrogen independent breast BT-20 and androgen independent prostate PC-3 cancer cells have been reported [22].

Heteroleptic complexes play an important role in biological processes, which are evident from the activation of enzymes by metal ions [23]. 2,2′-Bipyridine and 1,10 phenanthroline as co-ligands act as potential antitumor agents [24] and if these chelating agents are masked by copper(II) ions they can exhibit even better anticancer activity.

Sigman et al. [25] have first reported the DNA cleavage ability of copper(II) complexes of 1,10 phenanthroline. Since then heteroleptic copper(II) complexes containing diimines as co-ligands have been explored extensively for their strong interactions with DNA and cytotoxicity and antiviral activities [26–28].

Human breast cancer cell line (MDA-MB-231) belongs to triple-negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PR) expression and human epidermal growth factor receptor-2 (HER2). Among the 1.38 million new cases per year, more than 15% are designated to be TNBC, which has no efficient treatment. Dealing with the scarcity of well-defined molecular targets is still a challenge as its prognosis remains bleak [29]. Since, breast cancer therapy does not provide any effective drugs on TNBC, it is desirable to develop novel cytotoxic drugs for the treatment of TNBC. Hence, our strategy was to synthesize heteroleptic copper(II) complexes using three pyridazine-based Schiff base tridentate ligands and diimines (2,2′-bipyridine (bpy) and 1,10 phenanthroline (phen)) as co-ligands, and to assess their *in vitro* anticancer activity against one human breast cancer (MDA-MB-231) and one myoblast normal (L6) cell lines by MTT assay. The apoptotic study was carried out using AO/EB staining method and the cell cycle arrest was performed by flow cytometry.

2. Experimental section

2.1. Materials and instrumentation

2,2′-Bipyridine, 1,10-phenanthroline, 3,6-dichloropyridazine, salicylaldehyde, 5-bromosalicylaldehye and 4-(diethylamino)salicylaldehyde were purchased from Aldrich and used without further purification. Copper(II) carbonate was purchased from Fischer Scientific-Qualigens, India. Copper(II) perchlorate hexahydrate was prepared by the reaction of 70% perchloric acid with copper(II) carbonate. Solvents used in the synthesis were dried and purified before being used according to standard procedure [30]. The ligands 3-chloro-6-(salicylidenehydrazinyl)pyridazine (HL^1), 3-chloro-6-(4-diethylaminosalicylidenehydrazinyl)pyridazine (HL^2) and 3-chloro-6-(5-bromosalicylidenehydrazinyl)pyridazine (HL^3) were prepared according to the procedure reported in our earlier publication [7].

The melting points were obtained on an Electro thermal capillary apparatus and are uncorrected. Elemental analyses for C, H and N were obtained on a Carlo Erba model 1106 elemental analyzer. IR spectra were recorded on a Perkin-Elmer 297 spectrophotometer in the range 4000–400 cm^{-1}. ^1H and ^{13}C NMR spectra were recorded on an Avans Bruker 400 MHz spectrometer using DMSO-d_6 as solvent. ESI mass spectra were recorded on a Q-Tof mass spectrometer. Electronic spectra were recorded on a Perkin-Elmer Lambda 25 version 1.27 spectrophotometer using DMSO as solvent. EPR spectra (X-Band) were recorded on a Varian EPR-E 112 spectrometer using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) as the reference at 25 °C. The room temperature magnetic moment of the complexes have been recorded on a PAR (model-155) vibrating sample magnetometer. Elico digital conductivity bridge model CM-88 was used to measure molar conductance using a freshly prepared DMF solution of the complexes. Cyclic voltammograms were obtained on CHI 602D (CH Instruments Co., UA) electrochemical analyzer under oxygen free conditions, equipped with a three-electrode cell consisting of a platinum wire counter electrode, glassy carbon working electrode and Ag/AgCl reference electrode (saturated KCl solution). A DMF solution of TBAP (0.1 M) as the supporting electrolyte and a ferrocene/ferrocenium (Fc/Fc^+) couple was used as an internal standard. The reported potentials were measured relative to the Ag/Ag^+ reference electrode and $E_{1/2}$ of the Fc/Fc^+ couple. The concentration of complex solutions were taken around 1.0×10^{-3} M and the scan rate was 100 mVs^{-1}.

Caution! Metal perchlorate salts with organic ligands should be handled with care as they can cause explosion.

2.2. Synthesis of pyridazine-based heteroleptic copper(II) complexes

The heteroleptic copper(II) complexes of pyridazine-based ligands (HL^{1-3}) and diimines (2,2′-bipyridine or 1,10-phenanthroline) were prepared by following the same procedure as given below: A methanolic solution (15 mL) of $Cu(ClO_4)_2{\cdot}6H_2O$ (0.37 g, 1.0 mmol) was added to the appropriate ligand (HL^{1-3}, 1.0 mmol) in 1:1 methanol/DMF (20 mL) and an equimolar amount of triethylamine with constant stirring for 30 min followed by diimine (2,2′-bipyridine (0.16 g, 1 mmol) or 1,10-phenanthroline (0.23 g, 1 mmol)) in methanol (15 mL). The stirring was continued for 1 h and refluxed on a water bath for additional 2 h. The content was filtered while hot, and the filtrate was allowed to stand at room temperature for few days. The solid complexes obtained were recrystallized using hot methanol.

$[Cu(L^1)(bpy)](ClO_4)$ (**1**)

Yield: 0.41 g (72%); Color: Greenish brown. Anal. Calc. for $C_{21}H_{16}N_6O_5Cl_2Cu$, (FW: 566.84): C, 44.50; H, 2.85; N, 14.83; Found: C, 44.46; H, 2.82; N, 14.82%. Selected IR data (cm^{-1}): 3117 υ(−NH), 1603 υ(−C═N), 1251 υ(Ar−O), 1568 υ(−N═N), 1073 & 621 $\upsilon(ClO_4^-$, uncoordinated). UV-Vis (DMSO) λ_{max} (nm) (ε ($M^{-1}cm^{-1}$)): 287 (13,650), 349 (4041), 485 (1372), 645 (6 3 5). ESI-MS (*m/z*): 466.04 ($[Cu(L^1)(bpy)]^+$; 100%). Conductance (Λ_M, Ω^{-1} cm^2 mol^{-1}) in DMF: 68. $g_{||}$ = 2.20, $g_\perp$ = 2.09. μ_{eff} = 1.84 B.M.

$[Cu(L^1)(phen)](ClO_4)$ (**2**)

Yield: 0.44 g (75%); Color: Greenish brown. Anal. Calc. for $C_{23}H_{16}N_6O_5Cl_2Cu$, (FW: 590.86): C, 46.75; H, 2.73; N, 14.22; Found: C, 46.71; H, 2.71; N, 14.20%. Selected IR data (cm^{-1}): 3064 υ(−NH), 1606 υ(−C═N), 1243 υ(Ar−O), 1541 υ(−N═N), 1083 & 624 $\upsilon(ClO_4^-$, uncoordinated). UV-Vis (DMSO) λ_{max} (nm) (ε ($M^{-1}cm^{-1}$)): 266 (14,089), 367 (4070), 456 (1319), 661 (7 8 0). ESI-MS (*m/z*): 490.04 ($[Cu(L^1)(phen)]^+$; 100%). Conductance (Λ_M, Ω^{-1} cm^2 mol^{-1}) in DMF: 81. $g_{||}$ = 2.22, $g_\perp$ = 2.09. μ_{eff} = 1.81 B.M.

$[Cu(L^2)(bpy)](ClO_4)$ (**3**)

Yield: 0.45 g (71%); Color: Greenish brown. Anal. Calc. for $C_{25}H_{25}N_7O_5Cl_2Cu$, (FW: 637.96): C, 47.07; H, 3.95; N, 15.37; Found: C, 47.03; H, 3.92; N, 15.36%. Selected IR data (cm^{-1}): 3121 υ(−NH), 1612 υ(−C═N), 1245 υ(Ar−O), 1577 υ(−N═N), 1090 & 623 $\upsilon(ClO_4^-$, uncoordinated). UV-Vis (DMSO) λ_{max} (nm) (ε ($M^{-1}cm^{-1}$)): 273 (13,500), 355 (5450), 410 (1215), 642 (5 9 9). ESI-MS (*m/z*): 537.11 ($[Cu(L^2)(bpy)]^+$; 100%). Conductance (Λ_M, Ω^{-1} cm^2 mol^{-1}) in DMF: 73. $g_{||}$ = 2.22, $g_\perp$ = 2.09. μ_{eff} = 1.80 B.M.

$[Cu(L^2)(phen)](ClO_4)$ (**4**)