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Original article

Synthesis, structure and biological activity of copper(II) complexes of 4-(2-pyridylmethyl)-1,7-dimethyl-1,4,7-triazonane-2,6-dione and 4-(2-pyridylethyl)-1,7-dimethyl-1,4,7-triazonane-2,6-dione

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ABSTRACT

Four mononuclear copper(II) complexes **1**–**4** have been synthesized with two new *N*-functionalized macrocyclic ligands L^1 and L^2. All complexes are well characterized by various spectroscopic techniques, elemental analyses and conductivity measurements. Results suggest that Cu(II) ion has N_2O coordination from ligand and S_2 from two coordinated solvent molecules (S═CH_3CN for **1** and **3** while CH_3OH for **2** and **4**). The crystal structure of a representative complex **3** strengthen the proposed formulations for other isostructural copper(II) complexes. The structure of **3** shows few interesting features including rare *bent* mode of the coordinated CH_3CN molecules. All complexes were assayed for *in vitro* antimicrobial activity against clinically isolated resistant strains of *Pseudomonas aeruginosa* and *Proteus vulgaris*; and standard strains of *Staphylococcus aureus*, *P. aeruginosa*, *Klebsiella planticola* and *Escherichia coli*. Results indicate that the copper complexes possess notable antimicrobial properties with MIC values of 62.5–500 μg/ml. Studies on the U87 cancerous cell lines show potent cytotoxicity with IC_{50} and IC_{90} values of 2.9–93.5 and 30–250 μg/ml, respectively. In vitro toxicity tests demonstrate that all copper complexes are less cytotoxic than that of gentamycin on normal HEK cell lines. These copper complexes show the potential to act as antimicrobial and anticancer agent.

1. Introduction

The development of new anticancer and antimicrobial therapeutic agents is one of the fundamental goals in medicinal chemistry. One newer strategy for the research on new anticancer and antimicrobial therapeutic agents has been the use of metal containing compounds [1–5]. The unique properties of metal ions, such as versatile coordination geometries arising from multiple oxidation states, electron transfer abilities, and induced structural rigidity make them suitable candidates for potential medicinal applications [1–5]. An interesting example depicting the importance of the coordination complexes to place coordinated functional groups to interact with the enzyme active sites has been shown by Lebon et al. [6,7]. These authors have demonstrated the copper(II) complexes of the amide-based ligands with significant HIV-1 protease inhibition activity. The design aspects include the modeling of the catalytic water molecule to interact with the *Asp25* and *Asp125* residues; and amide C═O groups as proton acceptors to interact with *Ile50* and *Ile150* residues in the complex of HIV-1 [8]. These examples clearly demonstrate the unique ability of a coordination complex to place the ligated species in a certain orientation to interact with the inhibition sites.

Metal complexes can also act as complementary therapeutic agents to organic compounds that are widely sought in the drug discovery efforts [9]. In view of the emergence of drug-resistant cancer/microbial strains and undesirable side effects of drugs, there have been extensive worldwide efforts to develop new metal-based drug with fewer side effects to overcome the drug resistance [10]. Thus, the rational design of metal-based drugs has become increasingly important, and must continue to be so to make new advances in metallo-pharmaceutical research. In this context, copper complexes have attracted great deal of attention amongst the scientific community due to their therapeutic applications as hypoxia selectivity [11], anti-tumor [12,13], DNA cleavage [14], DNA binding [15,16], anti-diabetic [17], anti-oxidant [17–20], antimicrobial [18–22], anti-inflammatory [23] and radio-diagnostic activities [24]. Another advantage of using copper-based drugs is the possibility of employing radioactive isotopes, which can provide an additional anti-tumor mechanism of action [25].

Recently, we have shown the potent antibacterial activities of few cobalt(III) complexes with pyridine-amide-based ligands [26].

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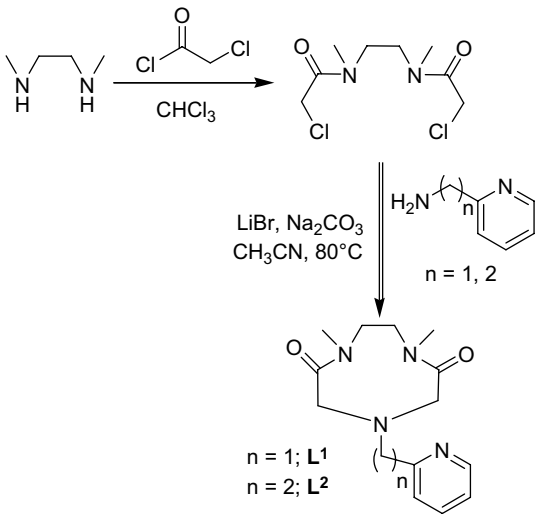
These compounds were designed in such a way to have un-coordinated pyridine rings that were speculated to interact with the cell membrane and/or the biologically important metal ions. The present study stems from our quest to prepare, characterize and evaluate the biological activities of the coordination compounds where a part of the complex/ligand has the ability to interact with the biologically important metal ions and/or cell membrane. The present copper(II) complexes have been synthesized with amide-based macrocyclic ligands having appended pyridine ring. The ligand coordinate the copper(II) ion via terminal N_{amine}, appended $N_{pyridine}$, and O_{amide} leaving behind one O_{amide} and two N_{amide} groups. The remaining two coordination sites are occupied by the labile solvent molecules onto the copper ion completing a distorted square-pyramidal geometry. We anticipate that the un-coordinated functional groups and the labile sites have the ability to interact with the cell membrane and/or the vital metal ions.

2. Results and discussion

2.1. Ligand design and synthesis

1,4,7-Triazacyclononane (TACN) based ligands have been extensively used as the chelating ligand to the metal ion in the field of coordination, bio-inorganic, organometallic, and oxidation chemistry [27]. However, the field of mono-functionalized TACN based ligands has remained under-explored due to the synthetic difficulty to prepare such ligands [27–31]. The literature synthetic procedures involve multiple steps involving selective protection and deprotection reactions and subsequently result in poor yield of the desired product [27–32]. Herein, we show a new synthetic route to prepare mono-functionalized TACN based ligands in fewer steps. The ligands L^1 and L^2 are synthesized in two steps starting from the *N*,*N*′-dimethylethylenediamine (Scheme 1). In the first step, diamine is converted into the bis(chloroacetamide) by treating with two equivalent of chloroacetylchloride. In the next step, the resultant bis(chloroacetamide) is treated with the corresponding pyridyl-amine to afford the desired ligand. Both the ligands are thoroughly characterized using various spectroscopic measurements and give satisfactory microanalysis results. This is worthwhile to mention here that the further reduction of the amide-based ligands L^1 and L^2 could afford the desired mono-functionalized TACN ligands in only three steps [33].



Scheme 1. Synthesis of ligands.

2.2. Synthesis and characterization of copper(II) complexes

Copper complexes, **1**–**4** were synthesized by the reaction of the ligand L^1 or L^2 with $Cu(ClO_4)_2 \cdot 6H_2O$ salt (Scheme 2). The use of CH_3CN or CH_3OH afforded complexes **1** and **3** or **2** and **4**, respectively. All copper(II) complexes were isolated as blue colored crystalline products in good recrystallized yield. The FTIR spectra of complexes **1** and **3** show $\nu_{C\equiv N}$ absorptions in the range of 2269–2314 cm^{-1} due to the coordinated nature of the CH_3CN molecule. Stretches due to the coordinated CH_3CN molecule have been reported to be observed in the similar region [34]. Similarly, complexes **2** and **4** display ν_{OH} and ν_{O-CH3} absorptions in the region of 3400–3500 cm^{-1} and ~1500 cm^{-1}, respectively, and suggest the presence of the coordinated CH_3OH molecule [34]. The FTIR spectra of all complexes also show intense $\nu_{C=O}$ features between 1615 and 1645 cm^{-1} due to the amide-II band. The ClO_4^- stretches were also noticed at ~1090 and ~620 cm^{-1} indicative of ionic nature of the anion. The solution conductivity data confirms the 1:2 electrolytic nature for all complexes [35], whereas the elemental analysis authenticates the purity of the bulk sample. In the mass spectra, molecular ion peak corresponding to the complexes **1**, **2**, **3**, and **4** were obtained at 621.46, 602.86, 634.27, and 616.73, respectively, and support the proposed formulations as well as the stability in the solution state. The labile nature of the coordinated solvent molecules was investigated by the exchange reactions between complexes. Complexes **1** and **3** can be inter-converted to **2** and **4**, respectively, by simple change of the solvent of study, CH_3CN or CH_3OH. This exchange study indicates that the present copper complexes have the tendency to bind a suitable solvent or ligand *via* exchange of the coordinated solvent molecules.

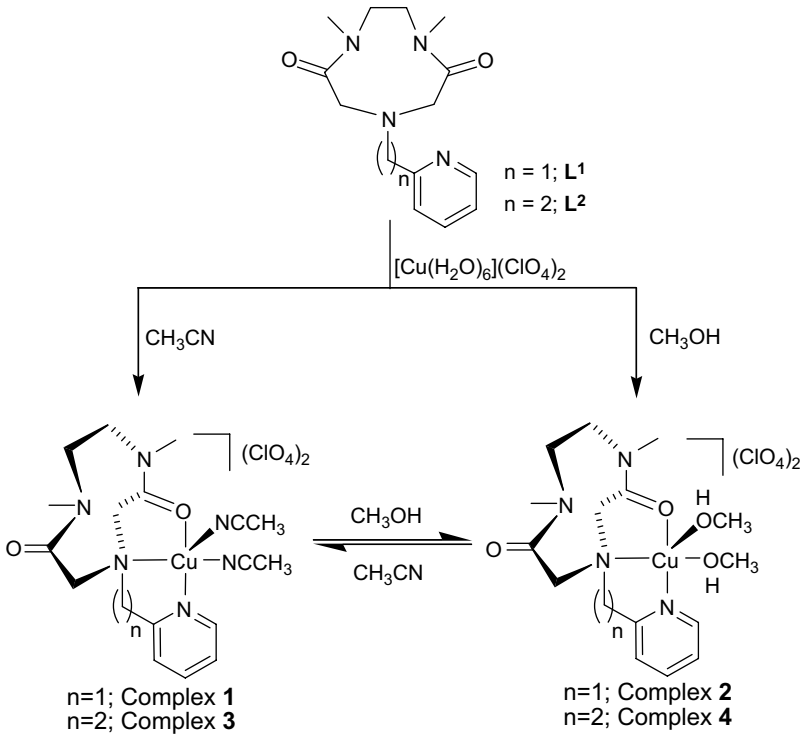
2.3. Absorption spectra

The absorption spectra of complexes **1** and **3** in CH_3CN display a broad band in the visible region at ~680 nm. Similarly, complexes **2** and **4** in CH_3OH show a broad feature at ~700 nm. The broadness of the absorption maxima and low intensity are suggestive of d–d transition nature of the electronic spectra. For an octahedral copper(II) complex, the ^2E_g to $^2T_{2g}$ transition is observed at ~800 nm. The λ_{max} observed in the present complexes show a considerable high energy shift due to the distortion of the octahedral geometry to square-pyramidal [36,37]. The high energy feature observed at ~330 nm and below could tentatively be assigned as metal-to-ligand and intra-ligand charge–transfer transitions. The solid state absorption spectra of the copper complexes indicate certain changes in the λ_{max} compared to that in solvent (CH_3CN or CH_3OH). In particular, the absorption maxima (λ_{max}) are *blue-shifted* moving from the solid to solution state for all complexes, thus indicates the dissociation of one or more coordinated solvent molecules in the solution.

The absorption spectra of all copper complexes were also recorded in the phosphate buffer saline (PBS) solution and display similar features as that of CH_3CN and CH_3OH. This study indicates that the copper complexes maintain their structural integrity in the PBS solution. This information will be helpful for the biological studies of the copper complexes in the PBS solution (*vide infra*).

2.4. Crystal structure study

One of the representative compounds, complex **3**, has also been characterized by the single crystal diffraction studies. The complex **3** contains a dipositive cation counterbalanced with two ClO_4^- anions. The molecular structure of the cationic part of the complex **3** is shown in Fig. 1 while the important crystallographic and structural details are shown in Tables 1 and 2, respectively. The copper ion is coordinated by the ligand L^2 in *pseudo-meridional* fashion *via* pyridine nitrogen atom N1 [1.978(6) Å], tertiary



Scheme 2. Synthesis of copper complexes.

nitrogen atom N2 [2.080(5) Å], and the amide oxygen atom O2 [1.966(5) Å]. The observed Cu–$N_{pyridine}$ and Cu–O_{amide} distances are quite on the lower side than the similar distances in other structurally characterized complexes [6,7,38–41]. It is important to mention here that the second O_{amide} group as well as both N_{amide} groups remains un-coordinated. In the 5-coordinate copper(II) center, three coordination sites are provided by the ligand L^2, whereas remaining two sites are occupied by two solvent molecules, CH_3CN. Two CH_3CN molecules coordinate asymmetrically with distances of 2.025(6) and 2.224(7) Å, respectively. The geometry around the copper(II) ion is best described as square-pyramidal with a very small trigonal–bipyramidal distortion parameter (τ) of 0.042 [$\tau = (\beta - \alpha)/60$, with α and β being the two largest coordination angles, 168.1° and 165.6°, respectively]. In a perfect square-pyramidal geometry τ equals 0, while it is 1 in a perfect trigonal–bipyramidal geometry [42]. The Cu(II) ion is 0.203 Å out of the basal plane defined by N1, N2, O2, and N5 towards the apical nitrogen atom (N6) of the coordinated CH_3CN. Such a displacement is quite common in square-pyramidal copper(II) complexes [43–45].

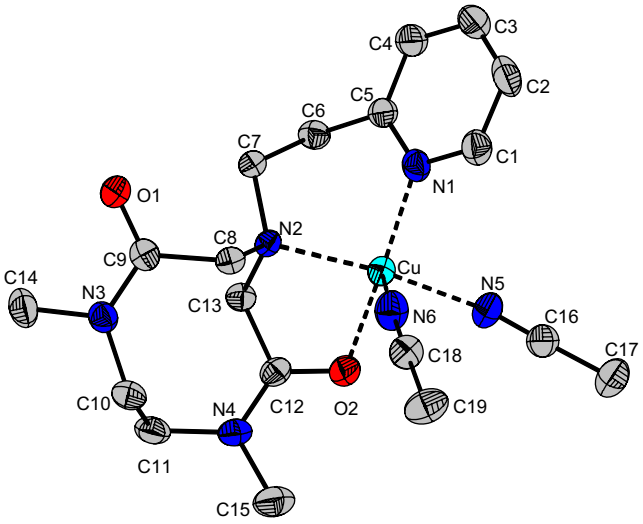


Fig. 1. Molecular structure of complex **3**. Thermal ellipsoids are drawn at 30% probability level while the hydrogen atoms and anions are omitted for clarity.

Table 1
Crystallographic and data collection parameters for $[Cu(L^2)(CH_3CN)_2](ClO_4)_2$ (**3**)

	$[Cu(L^2)(CH_3CN)_2](ClO_4)_2$ (**3**)
CCDC no.	686822
Empirical formula	$C_{19}H_{28}N_6O_{10}Cl_2Cu$
Mass	634.91
T [K]	295(2)
Crystal system	Triclinic
Space group	$P\bar{1}$
Colour, shape	Blue, block
Size [mm]	0.2 × 0.1 × 0.1
Theta range	1.84–25.49°
Index ranges	$0 \le h \le 12$, $-13 \le k \le 13$, $-15 \le l \le 14$
a [Å]	10.336(1)
b [Å]	12.343(1)
c [Å]	12.661(2)
α [°]	107.26(1)
β [°]	111.68(1)
γ [°]	101.09(1)
V [Å^3]	1347.9(3)
Z	2
F [000]	654
Reflections collected	5123
Unique reflections	4831
Observed reflections [$I > 2\sigma(I)$]	3053
D_{calcd} [g cm^{-3}]	1.564
Absorption coefficient [mm^{-1}]	1.071
R[a]	0.0663
R_w[b]	0.1712
GOF on F^2	1.012

[a] $R = \sum |F_o| - |F_c| / \sum |F_o|$.
[b] $R_w = \{[\sum(|F_o|^2 |F_c|^2)^2]\}^{1/2}$.

Table 2
Selected bond distances (Å) and angles (°) for $[Cu(L^2)(CH_3CN)_2](ClO_4)_2$ (**3**)

Bond distances	
Cu–N1	1.978(6)
Cu–N2	2.080(5)
Cu–N5	2.025(6)
Cu–N6	2.224(7)
Cu–O2	1.966(5)
Bond angles	
O2–Cu–N1	165.6(2)
O2–Cu–N5	87.6(2)
N1–Cu–N5	92.4(2)
O2–Cu–N2	83.35(19)
N1–Cu–N2	94.4(2)
N5–Cu–N2	168.1(2)
O2–Cu–N6	90.4(2)
N1–Cu–N6	104.0(2)
N5–Cu–N6	87.1(3)
N2–Cu–N6	100.7(2)
Cu–N5–C16	159.7(7)
Cu–N6–C18	155.1(6)
N5–C16–C17	178.0(9)
N6–C18–C19	178.4(9)
Dihedral angles	
N2–C13–C12–O2	−51.52
N2–C7–C6–C5	−77.08
N1–C5–C6–C7	57.98
Cu–N5–C16–C17	−60.94
Cu–N6–C18-C19	14.67

The N2–Cu–O2 bite angle (83.35°) is ∼11° smaller than the N1–Cu–N2 bite angle (94.4°) due to the involvement of a tight 5-membered chelate ring than the 6-membered for the later. The 5-membered chelate ring (defined by Cu, O2, C12, C13, and N2) makes an angle of 37.26° with the 6-membered chelate ring (defined by Cu, N1, C5, C6, C7, and N2). The 6-membered chelate ring is adopting an *envelope* conformation as seen by the relative angle of ∼85° between two half planes (between C5–C6–C7 and N1–Cu–N2). The 5-membered chelate ring is non-planar as observed by the large dihedral angle of ∼52° for the N2–C13–C12-O2 fragment. In a similar fashion, the 6-membered chelate ring is also non-planar (see dihedral angles, Table 2). Interestingly, two coordinated solvent molecules are non-linear in nature as reflected by their Cu–N≡C bond angles of 159.74° and 155.04°, for the equatorial and axial acetonitrile, respectively (Table 2). Examples of structurally characterized nitrile adducts having bent M–N≡C are extremely rare [46,47]. The observed deviation of the M–N≡C bond angle from 180° is within ∼8° for adducts of monodentate nitriles and ∼12° for compounds of chelating dinitriles [48–52]. To the best of our knowledge, only very few compounds have been reported so far having a similar M–N≡C angle as observed in this work [47–52].

The coordination of the O_{amide} group (O2) to the copper ion has resulted in the diminished resonance structure of the amide group that results in a longer C–O bond length [C12–O2: 1.248(8) Å] than the un-altered C–O group [C9–O1: 1.235(8) Å]. This resonance effect has also altered the C–N bond distances of the amide group with different bond lengths, [C9–N3: 1.359(8) Å and C12–N4: 1.311(8) Å, respectively].

2.5. Antibacterial studies

In the current study, all synthesized copper complexes have been tested against pathogenic clinically isolated resistant strains of *Pseudomonas aeruginosa* and *Proteus vulgaris*; and standard strains of *Staphylococcus aureus* (SA 96), *P. aeruginosa* (MTCC 1688), *Klebsiella planticola* (MTCC 2272) and *Escherichia coli* (T7) using the microbroth dilution method (Table 3) [53]. Gentamycin was used as a reference drug for these studies. Complexes **1**–**3** show good activity against resistant strain of *Proteus* and standard strain of *E. coli* (T7) with MIC value ranging from 62.5 to 125 μg/ml. Complex **4** appeared to have broad spectrum as it exhibit mild to moderate activity towards most of the strains. This study suggests that these complexes can further be explored as specific antibacterial drugs due to their decent activity against all experimental strains. Furthermore, complexes **1**–**4** also exhibit strong inhibitory characteristics against clinically isolated resistant strains as compared to gentamycin. We postulate that the effectiveness of the copper complexes could either be due to the available labile site or uncoordinated functional groups present in the complexes.

2.6. Cytotoxicity studies on cancerous U87 and normal HEK cell lines

The cytotoxicity studies for all copper complexes were also carried out. The study was used to test the growth inhibition by the MTT assay and the data are expressed in terms of % viability which is directly proportional to the metabolic active cell number. Copper complexes were found to be less cytotoxic than that of gentamycin on the normal HEK cells (Figs. 2 and 3). Cytotoxicity studies on the U87 cancerous cell lines show that all complexes are potent cytotoxic with their IC_{90} vales ranging from 30 to 250 μg/ml (Figs. 4–6, Table 4). The results of the cytotoxic activity of the metal complexes on the HEK cell lines suggest that the complexes are less cytotoxic at moderate concentrations (250–0.45 μg/ml). The importance of such work lies in the possibility that the next generation metal complexes might be more efficacious as antibacterial and anticancer agents. However, a thorough investigation relating the structure and the activity of the complexes as well as their stability under biological conditions is required. These detailed investigations could be helpful in designing more potent antibacterial and anticancer agents for the therapeutic use.

3. Conclusion

The present work demonstrates the synthesis of two amide-based mono-functionalized macrocyclic ligands and their coordination behaviour towards copper(II) ion. The copper(II) ion is

Table 3
Minimum inhibitory concentrations of Cu(II) complexes on resistant and standard bacterial strains by microbroth dilution method

Complex	Standard strains		MIC (μg/ml)		Resistant strains	
	Staphylococcus	*Pseudomonas*	*Klebsiella*	*Escherichia coli* (T7)	*Proteus*	*Pseudomonas*
1	250	250	–[a]	62.5	125	250
2	250	500	500	125	62.5	500
3	250	250	500	125	62.5	250
4	250	250	500	125	125	500
G[b]	15.6	7.8	250	7.8	3.9	125

Comparison of antimicrobial activities of Cu(II) complexes with gentamycin (G) against resistant strains of *Pseudomonas aeruginosa* and *Proteus vulgaris*; and standard strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella planticola* and *Escherichia coli* (T7).
[a] No activity.
[b] Gentamycin.

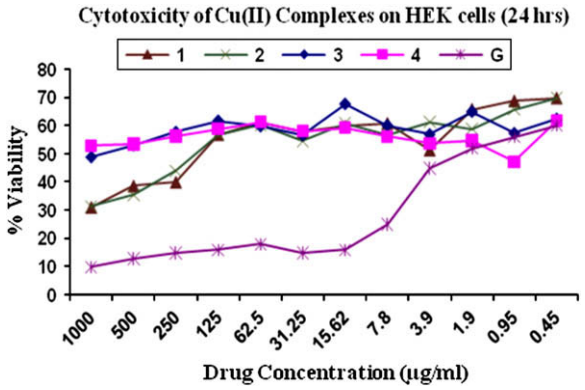


Fig. 2. Cytotoxicity of Cu(II) complexes on HEK cell line analysed by MTT assay at different concentrations after 24 h of treatment. Results are shown as the % viability. G stands for gentamycin.

coordinated by the ligands in a *pseudo-meridional* fashion *via* $N_{pyridine}$, terminal N_{amine}, and O_{amide} atoms while the second O_{amide} as well as both N_{amide} groups remain un-coordinated. Copper complexes have been thoroughly characterized by various physico-chemical investigations. One of the representative complexes, compound **3** has also been characterized crystallographically. The crystal structure shows square-pyramidal geometry around the Cu(II) center where N_2O coordinations are provided by the ligand L^2 while the remaining two sites are occupied by the solvent molecules. The coordinated solvent molecules are labial in nature as demonstrated by the exchange reactions between Cu–$NCCH_3$ and Cu–$O(H)CCH_3$ complexes. The antibacterial studies of these copper complexes show significant activity with MIC ranged from 62.5 to 500 μg/ml. The cytotoxicity studies on the U87 cancerous cell lines showed that all complexes are potent cytotoxic with their IC_{90} values from 30 to 250 μg/ml. On dilution activity decreases, which show that, the complexes are an effective inhibitor at moderate concentrations. The copper complexes were also tested for their cytotoxic activity on the normal HEK cell lines and reveal less cytotoxicity at all tested concentrations when compared with the gentamycin.

4. Experimental

4.1. Materials and methods

The chemicals and reagents were obtained from the commercial sources and used as received unless otherwise stated. Fetal calf serum (FCS), phosphate-buffered saline (PBS) and DMEM were obtained from the Hyclone, USA, whereas DMSO (cell culture tested) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from SRL, India. Acetonitrile (CH_3CN) was dried by distillation from anhydrous CaH_2. Diethyl ether was dried by refluxing over sodium metal under inert atmosphere. Methanol (CH_3OH) and ethanol (C_2H_5OH) were distilled from magnesium methoxide and magnesium ethoxide, respectively. Chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2) were purified by washing with 5% sodium carbonate solution followed by water and finally dried over anhydrous $CaCl_2$, before a final reflux and distillation.

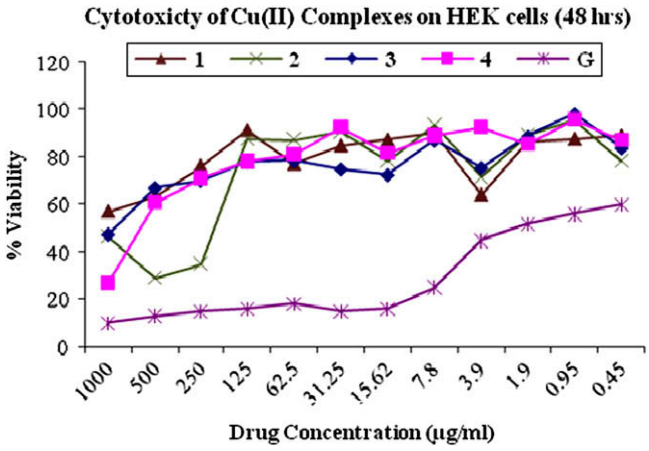


Fig. 3. Cytotoxicity of Cu(II) complexes on HEK cell line analysed by MTT assay at different concentrations after 48 h of treatment. Results are shown as the % viability. G stands for gentamycin.

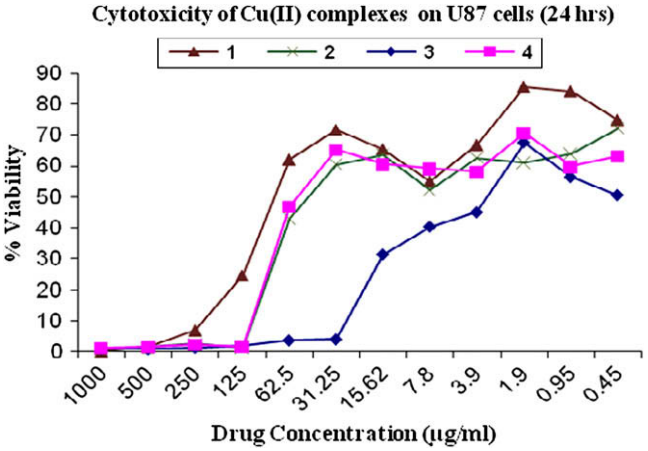


Fig. 4. Cytotoxicity of Cu(II) complexes on U87 cell line analysed by MTT assay at different concentrations after 24 h of treatment. Results are shown as the % viability.

4.2. Physical measurements

Solution electrical conductivity measurement was carried out with a digital conductivity bridge from Popular Trader, India (Model Number: PT-825). Elemental analysis data were obtained from the Elementar Analysensysteme Gmbh vario EL-III instrument. NMR measurements were done using an Avance Bruker 300 MHz instrument. Infrared spectra (either as KBr pellet or as mull in mineral oil) were recorded using Perkin-Elmer FTIR-2000 spectrometer. Absorption spectra were recorded using Perkin–Elmer *Lambda-25* spectrophotometer. ESI-MS mass spectra were obtained from the LC-TOF (KC-455) mass spectrometer of Waters.

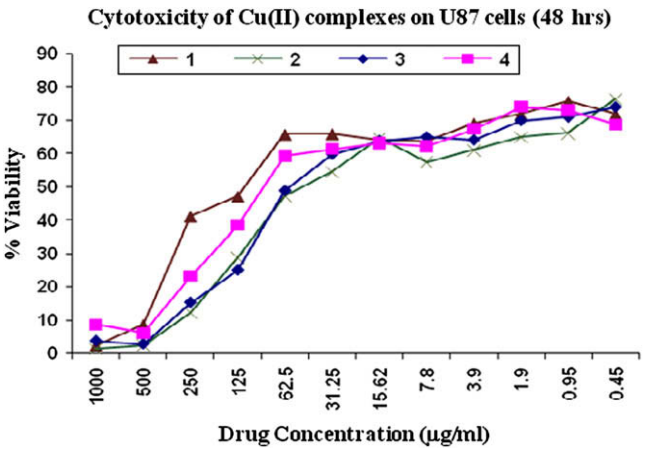


Fig. 5. Cytotoxicity of Cu(II) complexes on U87 cell line analysed by MTT assay at different concentrations after 48 h of treatment. Results are shown as the % viability.

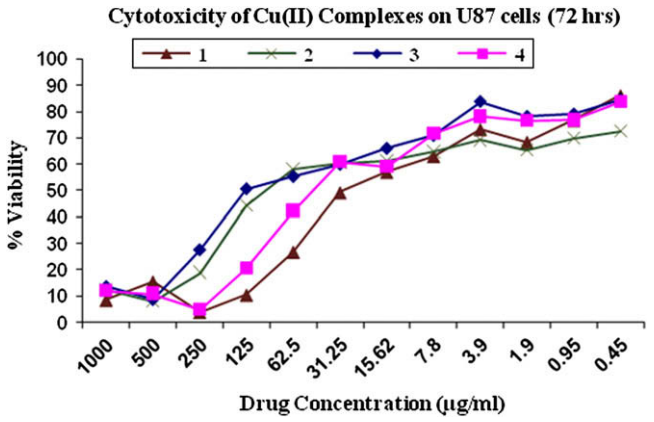


Fig. 6. Cytotoxicity of Cu(II) complexes on U87 cell line analysed by MTT assay at different concentrations after 72 h of treatment. Results are shown as the % viability.

4.3. Crystallography

Single crystals suitable for X-ray diffraction studies were grown by the vapor diffusion of diethyl ether to a saturated MeOH solution of **3**. A suitable crystal of **3** was mounted lengthways with the largest dimension in a sealed capillary. The intensity data were collected at 295 K on a Siemens P4 X-ray diffractometer by using scanning mode with graphite monochromatized Mo Kα radiation. A total of 5123 reflections were measured of which 3053 reflections ($I > 2\sigma(I)$) were used in the structure refinement. The data was corrected for Lorentz and polarization effects and a psi-scan absorption correction was also applied. The structure was solved by direct methods and refined by full-matrix least-squares refinement techniques on F^2. All calculations were performed using SHELXTL-PC. All non-hydrogen atoms were refined anisotropically whereas all hydrogen atoms were attached geometrically. Details of the crystallographic data and structure solution details are given in Table 1.

4.4. Syntheses

Caution! Although no problems were encountered in this work but transition metal perchlorate salts are potentially explosive and should be handled with great care.

4.4.1. Ligand synthesis

4.4.1.1. Synthesis of N,N′*-bis(2-chloroacetyl)*-N,N′*-dimethylethylene diamine. N, N′*-Dimethylethylenediamine (2.0 g, 22.6 mmol) in dry $CHCl_3$ (100 mL) was stirred at 0 °C and a solution of chloroacetyl-chloride (5.6 g, 49.9 mmol) in dry $CHCl_3$ (10 mL) was added drop wise. The mixture was stirred for 2 h at room temperature followed by heating at reflux for another 4 h. The reaction mixture was then allowed to cool to room temperature and then washed with cold water (3 × 100 mL). The organic phase was collected and dried over Na_2SO_4. The solvent was removed under reduced pressure to obtain crude product as yellowish white solid. This product was recrystallized by layering $CHCl_3$ solution with hexanes to afford a white solid. Yield: 4.10 g, 75%. Mp: 125 °C. Anal. Calcd for $C_8H_{14}N_2O_2Cl_2$: C, 39.85, H, 5.85, N, 11.62. Found: C, 39.96; H, 5.91; N, 11.61. δ_H (300 MHz, $CDCl_3$, 25 °C, TMS): 3.13 (s, 6H, N–CH_3), 3.66 (s, 4H, N–CH_2), 4.09 (s, 4H, –CH_2Cl). IR (KBr, ν, selected peaks): 1654, 1180, and 1106 cm^{-1}.

4.4.1.2. Synthesis of 4-(2-pyridylmethyl)-1,7-dimethyl-1,4,7-triazo nane-2,6-dione (L^1). N, N′-Bis(2-chloroactyl)-N,N′-dimethylethylenediamine, (1.0 g, 4.1 mmol), LiBr (0.72 g, 8.2 mmol), and Na_2CO_3 (3.5 g, 32.8 mmol) were added to CH_3CN (150 mL) and the mixture was heated to reflux with stirring for 30 min. A solution of 2-aminomethylpyridine (0.44 g, 4.1 mmol) in CH_3CN (10 mL) was added drop wise to the aforementioned reaction mixture and the reflux was continued for another 48 h. The reaction mixture was cooled to room temperature and filtered through a pad of celite on a medium porosity frit. The filtrate was concentrated under reduced vacuum to afford a yellow viscous liquid which was purified by the column chromatography (silica gel, 1:9 $CH_3OH/CHCl_3$ as eluent). The desired product had an R_f value of 0.5 (1:9 $CH_3OH/CHCl_3$). Yield: 0.57 g, 50%. Anal. Calcd for $C_{14}H_{20}N_4O_2$: C, 60.85; H, 7.30; N, 20.28. Found: C, 60.92; H, 7.30; N, 20.90. δ_H (300 MHz, $CDCl_3$, 25 °C, TMS): 3.01 (s, 6H, CH_3), 3.47 (s, 4H, –CH_2CH_2–), 3.86 (s, 4H, –$COCH_2$), 4.00 (s, 2H, –CH_2Py), 7.23 (m, 1H, py-5), 7.37 (d, 1H, py-3), 7.71 (t, 1H, py-4), 8.58 (d, 1H, py-6). IR (KBr, ν, selected peaks): 1642 (C=O) cm^{-1}. MS (CH_3CN, m/z): 277.17 ($L^1 + H^+$), 200.34 (L^1 – Py), 149.02 ($L^1 - CH_2Py + (CH_3)_2$).

4.4.1.3. Synthesis of 4-(2-pyridylethyl)-1,7-dimethyl-1,4,7-triazon ane-2,6-dione (L^2). The synthesis of ligand L^2 was followed the similar procedure as that of the ligand L^1. The crude product was purified by the column chromatography (silica gel, 1:9 $CH_3OH/CHCl_3$ as eluent). The desired product had an R_f of 0.4 (1:9 CH_3OH/CH_3Cl). Yield: 0.62 g, 52%. Anal. Calcd for $C_{15}H_{22}N_4O_2$: C, 62.05; H, 7.64; N, 19.30. Found: C, 62.12; H, 7.85; N, 19.45. δ_H (300 MHz, $CDCl_3$, 25 °C, TMS): 2.93 (s, 6H, CH_3), 3.04 (m, 4H, –CH_2CH_2Py), 3.46 (s, 4H, –CH_2CH_2–), 3.57 (s, 4H, –$COCH_2$), 7.14 (m, 1H, py-5), 7.20 (m, 1H, py-3), 7.63 (t, J = 7.1 Hz, 1H, py-4), 8.52 (m, 1H, py-6). IR (KBr, ν, selected peaks): 1638 (C=O) cm^{-1}. MS (CH_3CN, m/z): 290.35 ($L^2 + H^+$), 262.45 ($L^2 - (CH_3)_2$), 169.66 ($L^2 - CH_2Py + (CH_3)_2$).

4.4.2. Synthesis of copper complexes

4.4.2.1. General synthetic procedure for the Cu(II) complexes. A solution of $Cu(ClO_4)_2 \cdot 6H_2O$ in CH_3CN or CH_3OH was added to the CH_3CN or CH_3OH solution of the respective ligand. The reaction mixture was stirred for 1 h at room temperature. The solution was filtered, followed by the removal of the solvent under reduced pressure. The crude compound was isolated after washing with Et_2O and was further recrystallized by the vapor diffusion of Et_2O to a saturated solution of the salt in CH_3CN or CH_3OH. This afforded blue colored crystalline material that was filtered and dried under vacuum.

*$[Cu^{II}(L^1)(CH_3CN)_2](ClO_4)_2$ (**1**).* Yield: 70%. Anal. Calcd for $C_{18}H_{26}N_6O_{10}CuCl_2$: C, 34.82; H, 4.22; N, 13.54. Found: C, 34.78; H, 4.21; N, 13.53. IR (KBr pellet, ν, selected bands): 2268–2314 (C≡N), 1643, 1619 (C=O), 1091 (ClO_4) cm^{-1}. $\lambda_{max/nm}$ (CH_3CN, ε, $dm^3\,mol^{-1}\,cm^{-1}$): 682 (140), 336 (sh, 1400), 258 (10200). MS (CH_3CN, m/z): 621.46 ($[CuL^1(CH_3CN)_2](ClO_4)_2 + H^+$), 439.95 ($[CuL^1(ClO_4)]^+$), 381.63 ($[CuL^1(CH_3CN)] - H^+$). Molar conductivity (~1 mM, CH_3CN, 25 °C): Λ = 260 S $cm^2\,mol^{-1}$ (the range for 1:2 electrolytes in CH_3CN is 220–260).

Table 4
IC_{50} and IC_{90} values after 24 h exposure to copper complexes (mean ± std. dev., std. error)

In vitro toxicity results	1	2	3	4
IC_{50} (µg/ml)				
U87	93.5 (±6.3, 4.5)	46.8 (±9.6, 6.8)	2.9 (±1.4, 1.0)	62.5 (±3.5, 2.5)
HEK	>1000	187.5 (±10.6, 7.5)	750 (±28.2, 20)	500 (±42.5, 42.4)
IC_{90} (µg/ml)				
U87	250 (±21.2, 15.0)	120 (±7.07, 5.0)	30 (±7.07, 5.0)	120 (±2.82, 2.0)
HEK	>1000	>1000	>1000	>1000

*$[Cu^{II}(L^1)\ (CH_3OH)_2](ClO_4)_2$ (**2**).* Yield: 65%. Anal. Calcd for $C_{16}H_{28}N_4O_{12}Cl_2Cu$: C, 31.88; H, 4.68; N, 9.29. Found: C, 31.69; H, 4.95; N, 9.90. IR (KBr pellet, ν, selected bands): ~3500 (O–H), 1630, 1615 (C=O), 1094 (ClO_4) cm^{-1}. $\lambda_{max/nm}$ (CH_3OH, ε, $dm^3\,mol^{-1}\,cm^{-1}$): 725 (120), 257 (11500). MS (CH_3OH, m/z): 603.58 ($[CuL^1(CH_3OH)_2](ClO_4)_2 + H^+$), 403.01 ($[CuL^1(CH_3OH)_2] - H^+$). Molar conductivity (~1 mM, CH_3OH, 25 °C): $\Lambda = 220\ S\,cm^2\,mol^{-1}$ (the range for 1:2 electrolytes in CH_3OH is 160–220).

*$[Cu^{II}(L^2)\ (CH_3CN)_2](ClO_4)_2$ (**3**).* Yield: 72%. Anal. Calcd for $C_{19}H_{28}N_6O_{10}Cl_2Cu.H_2O$ (including one water molecule): C, 34.95; H, 4.63; N 12.87. Found: C, 34.84; H, 4.81; N, 12.56. IR (KBr pellet, ν, selected bands): 2269–2314 (C≡N), 1640, 1614 (C=O), 1091 (ClO_4) cm^{-1}. $\lambda_{max/nm}$ (CH_3CN, ε, $dm^3\,mol^{-1}\,cm^{-1}$): 664 (130), 329 (1600), 261 (9800). MS (CH_3CN, m/z): 635.43 ($[CuL^2(CH_3CN)_2](ClO_4)_2 + H^+$), 593.24 ($[CuL^2(CH_3CN)](ClO_4)_2 + H^+$), 452.76 ($[CuL^2(ClO_4)]^+$). Molar conductivity (~1 mM, CH_3CN, 25 °C): $\Lambda = 245\ S\,cm^2\,mol^{-1}$ (the range for 1:2 electrolytes in CH_3CN is 220–260).

*$[Cu^{II}(L_2)\ (CH_3OH)_2](ClO_4)_2$ (**4**).* Yield: 68%. Anal. Calcd for $C_{17}H_{30}N_4O_{12}Cl_2Cu$: C, 33.10; H, 4.90; N, 9.08. Found: C, 33.29; H, 4.96; N, 9.38. IR (KBr pellet, ν, selected bands): 3413 (O–H), 1626, 1611 (C=O), 1089 (ClO_4) cm^{-1}. $\lambda_{max/nm}$ (CH_3OH, ε, $dm^3\,mol^{-1}\,cm^{-1}$): 705 (140), 258 (11600). MS (CH_3OH, m/z): 617.58 ($[CuL^2(CH_3OH)_2](ClO_4)_2 + H]^+$), 518.13 ($[CuL^2(CH_3OH)_2(ClO_4)]^+$). Molar conductivity (~1 mM, CH_3OH at 25 °C): $\Lambda = 215\ \Omega^{-1}\,S\,cm^2\,mol^{-1}$ (the range for 1:2 electrolytes in CH_3OH is 160–220).

4.5. In vitro antibacterial activity

All complexes have been screened *in vitro* against clinically isolated resistant strains of *P. aeruginosa* and *P. vulgaris*, *S. aureus*, *P. aeruginosa* (MTCC 1688), *K. planticola* (MTCC 2272) and *E. coli* (T7). Various methods [54–56] are available for the evaluation of the antibacterial activity of different types of drugs. However, the most widely used method [56], which consists of determining the antibacterial activity of the drug is to add it in known concentrations to the cultures of the test organisms, was employed.

4.5.1. Microbroth dilution assay

Different concentrations of the test compounds in 200 μL of culture medium were prepared in 96-well flat-bottomed microculture plates (Nunc, Nunclon) by double dilution method. The wells were prepared in triplicate for each concentration. Each well was inoculated with 10.0 μL of bacterial suspension containing 10^7 cells/ml. The plates were incubated at 37 °C for 16 h and the OD was measured at A_{600} nm of the suspension to assess the inhibition of the cell growth due to treatment with compounds. All tests were repeated minimum for 3 times.

4.6. In vitro cell growth inhibition assay

Cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well in 200 μL of complete media and incubated for 24, 48 and 72 h at 37 °C in 5% CO_2 atmosphere to allow for cell adhesion. Stock solutions (2 mg/ml) of the compounds made in water were filter-sterilized, then further diluted up to 0.45 μg/ml incomplete media for treatment against HEK and U87 cell lines. A 100 μL solution of compound was added to a 100 μL solution of fresh medium in wells to give final concentrations of 1000–0.45 μg/ml. All assays were performed in two independent sets of quadruplicate tests. Control group containing no drug was run in each assay. Following 24, 48 and 72 h exposure of cells to drug, each well was carefully rinsed with 200 μL PBS buffer. Cytotoxicity was assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) [57]. A 20 μL MTT solutions (5 mg mL^{-1} dd H_2O) along with 200 μL of fresh, complete media were added to each well and plates were incubated for 4 h. Following incubation, the medium was removed and the purple formazan precipitate in each well was sterilized in 200 μL DMSO. Absorbance was measured using Techan microplate reader (molecular device) at 570 nm and results are expressed as % viability which is directly proportional to metabolic active cell number. Percentage (%) viability was calculated as

$$\%\text{Viability} = \text{OD in sample well}/\text{OD in control well} \times 100.$$

5. Supplementary information

Crystallographic data for complex **3** has been deposited at the Cambridge Crystallographic Data Centre as supplementary publication (CCDC no. 686822). This data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK [fax: +44 1223/336 033; e-mail: deposit@ccdc.cam.ac.uk].

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