Synthesis, structure and anticancer activity of copper(II) complexes of N-benzyl-2-(diethylamino)acetamide and 2-(diethylamino)-N-phenylethylacetamide

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Received 30 October 2010; accepted 23 December 2011

The ligands N-benzyl-2-(diethylamino)acetamide, (HL\textsubscript{1}) and 2-(diethylamino)-N-phenylethylacetamide (HL\textsubscript{2}), have been used to synthesize copper(II) complexes, [Cu(HL\textsubscript{1})\textsubscript{2}](ClO\textsubscript{4})\textsubscript{2} (1) and [Cu(HL\textsubscript{2})\textsubscript{2}](ClO\textsubscript{4})\textsubscript{2} (2), respectively. Both complexes are well characterized by various spectral and physical methods. The crystal structure of complex (1) reveals that two bidentate ligands coordinate the Cu(II) ion via O\textsubscript{amide} and N\textsubscript{amine} atoms in the basal plane whereas one of the ClO\textsubscript{4} ions occupies the apical position maintaining a square–pyramidal geometry. Screening results for anti–proliferative studies against the U87 and HeLa cancerous cells indicate promising activity. The complexes enhanced growth inhibition and cell death in a concentration and time dependent manner for both U87 and HeLa cell lines. Of the two compounds, complex (2) exhibits better activity against both HeLa and U87 cells. Further, both complexes are specifically potent against U87 after 72 h of treatment. Micronucleus and apoptosis frequencies are 3 – 4 times higher in treated cells when compared with untreated control. Despite potent \textit{in vitro} activity, both complexes exhibit diminished cytotoxicity against the normal human HEK cells at all effective concentrations.

\textbf{Keywords}: Bioinorganic chemistry, Copper, Amide-based ligands, Anti-cancer activity, Cytotoxicity

The chemistry of transition metal complexes has received considerable attention largely due to their catalytic and bioinorganic relevance. Such complexes are also important due to their potential biological activities such as antibacterial, antiviral, antifungal, antimalarial and antitumor\textsuperscript{1-6}. A transition metal ion can coordinate a ligand in a precise three-dimensional configuration thus allowing the tailoring of the molecule to recognize and interact with a defined molecular target. This is further enhanced by the diversity of chemical modification of ligands and selection of metal ions. Transition metal ions possess different oxidation states which not only allows for modification of the three-dimensional space into which the molecule can fit, but significantly permits them to participate in biological redox chemistry\textsuperscript{7,8}. In addition, the ability to undergo ligand exchange reactions offers unique opportunities for metal ions to interact and coordinate with biologically important ions and molecules.

Medicinal inorganic chemistry is comparatively a new discipline, which developed after the serendipitous discovery of the anti-tumor activity of \textit{cis}-platin\textsuperscript{9}. The clinical success of this platinum complex has stimulated considerable interest in the search for new metal complexes as modern therapeutics, diagnostic and radiopharmaceutical agents. In this direction, copper and zinc complexes are used in the treatment of many diseases including cancer\textsuperscript{10-12}, cobalt complexes have been investigated as potential hypoxia-activated pro-drugs\textsuperscript{13-20}, whereas chromium, manganese and iron complexes have been used for antibacterial activity\textsuperscript{21}. Zinc is essential for the structure, regulation and catalytic action of over 300 enzymes\textsuperscript{22,23}. It is a well known fact that the biological properties of metals are determined both by speciation and the ligand present around the metal center. For example, the simple chloride salt of platinum, such as [PtCl\textsubscript{2}]\textsuperscript{2+}, are known as sensitizers eliciting a potential fatal allergic reaction, whereas the neutral complex \textit{cis}-dichlorodiamine platinum(II) complex, [Pt(NH\textsubscript{3})\textsubscript{2}(Cl)\textsubscript{2}], is one of the most successful cancer drugs of recent years\textsuperscript{24}. One of the potential approaches in anticancer chemistry is focused on the design of new metal compounds with different substituents and labile sites which may increase their cytotoxicity, specifically to cancer cells. In this context, the wide range of coordination numbers and geometries, accessible redox states, thermodynamic and kinetic characteristics, and the
intrinsic properties of the cationic metal ion and ligand itself offer the medicinal chemist a wide spectrum of reactivities that can be exploited.

We are also interested in preparing biologically active coordination complexes containing either uncoordinated functional groups and/or labile sites capable of interacting with vital metal ions and/or cell membrane of the bacterium cell. Recently, we have described the synthesis and characterization of Co(II) complexes of pyridine-amide ligands and anticancer activities of two copper(II) complexes supported with bidentate amide-based ligands and their further utilization as the building blocks. These Co(II) complexes were shown to have encouraging antimicrobial activity and anticancer properties. Interestingly, out of several complexes, two complexes showed very high antimicrobial activity due to the presence of uncoordinated functional groups potentially capable of interacting with biologically important metal ions required for the functioning of the bacterium cell. More recently, few Cu(II) complexes supported with amide–based macrocyclic ligands containing labile sites were shown to have good anticancer properties. The present work is in continuation of our search for coordination complexes, where a part of the molecule will offer uncoordinated functional groups and/or labile sites and evaluating their biological activity. Specifically, we show the synthesis, characterization and anticancer activities of two copper(II) complexes supported with bidentate amide-based ligands.

Materials and Methods

The reagents and chemicals were obtained from the commercial sources and used as received. Solvents were purified as reported earlier. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide), Hoechst-33342 (bis-benzimide), HEPES (N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]) buffer, propidium iodide (PI), ribonuclease-A (RNase-A) and trypsin were obtained from Sigma Chemical Co., USA.

The conductivity measurements were made in organic solvents using the digital conductivity bridge from the Popular Traders, India (model PT-825). The elemental analyses data were obtained on Elemental Analysen Systeme GmbH Vario EL-III instrument. The NMR spectra were recorded on Avance Bruker (300 MHz) instrument. The infra red spectra (either as KBr pellet or as a mull in mineral oil) were recorded using Perkin-Elmer FTIR-2000 spectrometer. The absorption spectra were recorded using Perkin–Elmer Lambda-25 spectrophotometer. The mass spectra were obtained on LC-TOF (KC-455) mass spectrometer of Waters. Solution magnetic susceptibility measurements were made by the Evans’ NMR method with a Hitachi R-600 FT NMR (60 MHz) spectrometer. The diamagnetic corrections were applied according to the literature.

Single crystals suitable for the X-ray diffraction studies were grown by the vapour diffusion of diethyl ether to a CH₃CN solution of complex (I). The intensity data for complex (I) were collected at 293(2) K on a Bruker AXS Smart-Ax CCD diffractometer equipped with a molybdenum sealed tube (Mo-Kα = 0.71073 Å) and a highly orientated graphite monochromator. Frames were collected by ω, φ and 2θ rotation at 10 s per frame with SMART. A total of 18832 reflections were measured of which 6859 [I > 2σ(I)] were considered observed. The measured intensities were reduced to F² and corrected for absorption with SADABS. The structure was solved by SIR 92 expanded by Fourier difference syntheses and refined with the SHELXL 97 package incorporated in WINGX 1.70 crystallographic collective package. The positions of the hydrogen atoms were calculated by assuming ideal geometries. All hydrogen atoms were fixed at the calculated positions with isotropic thermal parameters and all non-hydrogen atoms were refined anisotropically by full-matrix least-squares procedures on F². Intermolecular interactions were examined with the DIAMOND 2.0 and 3.0 packages. Details of the crystallographic data collection and structure solution parameters are given in Table 1.

Synthesis of the ligands

Both ligands were synthesized in two steps; in the first step, the respective chloroacetamide was prepared that was further reacted with diethyl amine in the second step to afford the desired ligand.

N-benzyl-2-chloroacetamide

An ice cooled solution of chloroacetyl chloride (0.75 ml, 9.33 mmol) in 5 ml THF was added dropwise to a stirred solution of benzyl amine (1.0 g, 9.33 mmol) and triethylamine (1.41 g, 13.99 mmol) in 10 ml THF. After the addition was complete, the mixture was stirred for 1 h on ice bath and additional 2 h at room temperature. After that the volatiles were
removed under reduced pressure to afford a brown solid. This solid was dissolved in minimum amount of chloroform and washed with water (10 mL × 3). The organic layer was separated, dried over Na₂SO₄ and concentrated to one-third of its original volume. The CHCl₃ solution was layered with hexanes to afford a white crystalline compound that was filtered, washed with hexanes and dried over CaCl₂ under vacuum. Yield: 1.20 g (70 %). ¹H NMR (300 MHz, CDCl₃): δ 4.07 (2H, s, -CH₂), 4.50 (2H, d, PhCH₂NH-), 6.95 (1H, s, NH), 7.26 – 7.38 (5H, m, -C₆H₅). FTIR (Nujol, cm⁻¹): ν (NH) 3278, ν(C=O) 1468, ν(C-H) 2970, 2934, 2872, 1458, 1433, 1378, 1366, 1206, 1139, 1055. Mass (EI, ClO₄): m/z 476, 475, 473, 472, 471, 470, 469, 468, 234. Anal. (%): Calc. for C₂₅H₂₄N₄O₄Cl₂: C, 55.46; H, 4.43; N, 10.30. Found: C, 55.38; H, 4.39; N, 10.28.

N-benzyl-2-(diethylamino)acetamide (HL¹)
A mixture of N-benzyl-2-chloroacetamide (1 g, 5.44 mmol), diethyl amine (0.70 g, 10.89 mmol) and K₂CO₃ (22.59 g, 169.48 mmol) were taken in 20 ml CH₃CN and refluxed for 12 h. After that K₂CO₃ was filtered off and volatiles were removed under reduced pressure to afford brown oil. Yield: 1.0 g (76 %). ¹H NMR (300 MHz, CDCl₃): δ 4.09 (6H, t, -CH₂), 2.5 (4H, q, -CH₂), 3.06 (2H, s, -NCH₂CO(O)-), 4.45 (2H, d, PhCH₂N-), 7.25 – 7.34 (5H, m, -C₆H₅), 7.85 (1H, s, NH). FTIR (NaCl plate, cm⁻¹): ν (NH) 3241, ν(C-H) 2970, 2934, ν(C=O) 1647, 1573, 1451, 1378, 1055. Mass (EI, CH₃OH, m/z): Calcd. for C₁₃H₁₄N₂O: 220.32. Anal. (%): Calc. for C₁₃H₁₄N₂O: C, 70.87; H, 9.15; N, 12.72. Found: C, 70.54; H, 9.00; N, 12.44.

2-Chloro-N-phenylethylacetamide
This compound was prepared in a similar manner as that of N-benzyl-2-chloroacetamide using chloroacetyl chloride (1.4 ml, 8.33 mmol), β-phenylethylamine (1.0 g, 8.33 mmol) and triethylamine (1.25 g, 12.88 mmol). Yield: 1.23 g (76 %). ¹H NMR (300 MHz, CDCl₃): δ 4.00 (2H, s, -CH₂Cl), 3.54 (2H, q, -CH₂NH), 2.85(2H, t, C₆H₅CH₂-), 6.6 (1H, s, NH), 7.19 – 7.35 (5H, m, -C₆H₅). FTIR (Nujol cm⁻¹) ν (NH) 3333, ν(C=O) 1649 cm⁻¹. M. pt. 64 ºC.

2-(Diethylamino)-N-phenylethylacetamide (HL²)
The ligand HL² was prepared in an analogous manner as that of ligand HL¹ using 2-chloro-N-phenylethylacetamide (1.0 g, 5.05 mmol), diethyl amine (0.74 g, 10.10 mmol), and K₂CO₃ (21.04 g, 151.50 mmol). The product was obtained as deep brown oil. Yield: 0.83 g (73 %). ¹H NMR (300 MHz, CDCl₃): δ 0.9 (6H, t, -CH₃), 2.44 (4H, q, -CHᵢ₂Nᵢ), 2.80(2H, m, -NHCH₂), 2.95(2H, s, -NCH₂CO(O)-), 3.56(2H, t, C₆H₅CH₂-), 7.17 – 7.27 (5H, m, -C₆H₅), 7.4 (1H, s, NH). FTIR (NaCl plate, cm⁻¹): ν (NH) 3346, ν (C-H) 2970, 2934, ν(C=O) 1669 cm⁻¹. Mass (EI, CH₃OH, m/z): Calcd. for C₁₃H₂₂N₂O: 234.34; Found 234.46. Anal. (%): Calc. for C₁₃H₂₂N₂O: C, 71.76; H, 9.46; N, 11.95. Found: C, 71.54; H, 9.60; N, 12.14.

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

General synthetic procedure for the copper(II) complexes
A solution of Cu(ClO₄)₂·6H₂O in CH₃OH was added drop-wise to a two equivalents CH₃OH solution of the respective ligands. The reaction mixture was stirred for 1.5 h at room temperature. The solution was filtered, followed by the removal of solvent under reduced pressure to afford deep blue solid. The crude product was isolated after washing with diethyl ether. The product was further re-crystallized by the vapor diffusion of Et₂O to a saturated solution of crude complex in CH₂CN. This afforded a highly crystalline product within 2 – 3 days which was isolated, washed with Et₂O and dried under vacuum.

[Cu(HL²)₂(ClO₄)₂(I)]
Yield: 83%. Anal. (%): Calc. for C₂₅H₄₀N₄O₁₆Cl₂Cu: C, 44.42; H, 5.73; N, 7.97. Found: C, 44.41; H, 5.70; N, 7.96. IR (Nujol, cm⁻¹): ν(NH) 3294; ν(C=O), 1632; ν (ClO₄) 1127, 1055. Conductivity (ΛM, CH₂CN): 300 Ω⁻¹ cm² mol⁻¹ (range

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Table 1—Crystallographic data collection and structure solution parameters for [Cu(HL²)₂(ClO₄)₂(I)]
in CH₂CN for 1:2 electrolytes: 220 – 300 Ω⁻¹ cm⁻² mol⁻¹). UV-vis: (Nujol; λ_max, nm): 620 (sh), 540, 340, 250. UV-vis (MeCN; λ_max, nm (ε, M⁻¹ cm⁻¹): 620 (120), 303 (3870), 240 (5770). μ_eff (MeCN, 298K, Evans’ method): 1.85 BM

[Cu(HL)₂](ClO₄)₂ (2)
Yield: 85 %. Anal. (%): Calc. for C₂₅H₄₈N₄O₁₀Cl₂Cu: C, 46.00; H, 6.07; N, 7.66. Found: C, 45.96; H, 5.89; N, 7.54. IR (KBr, cm⁻¹): ν(NH), 3296, ν(C=O), 1625 and ν(ClO₄⁻), 1110 (split). Conductivity (ΛM CH₂CN): 280 Ω⁻¹ cm² mol⁻¹ (range in CH₂CN for 1:2 electrolytes: 220 – 300 Ω⁻¹ cm⁻² mol⁻¹). UV-vis (Nujol; λ_max, nm): 775 (sh), 725 (sh), 680 (sh), 570 (sh). UV-vis (MeCN; λ_max, nm (ε, M⁻¹ cm⁻¹): 619 (105), 301 (3320), 240 (4750). μ_eff (MeCN, 298K, Evans’ method): 1.94 BM.

Human cell lines
Human cerebral glioma tumor U87, cervical cancer HeLa and normal HEK cells used in the present studies were purchased from the National Center for Cell Sciences (NCCS, Pune, India). All three cell lines were cultured in 75 cm² culture flasks (Corning, USA) using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % nonessential amino acids, 1 % glutamine, penicillin (100 IU/ml) and streptomycin (100 mg/ml) (all from Euroclone, UK). All cultures were maintained at 37 °C, 95 % relative humidity and 5 % CO₂. The growth medium was changed every other day until the time of use. The U87 cells of passage numbers 54-60, HeLa cells of passage numbers 31–34 and HEK cells passage number 47-49 were used in the cytotoxicity tests. Prior to each cytotoxicity test, the cells were harvested using trypsin–ethylenediamine tetraacetic acid (EDTA)–PBS solution (Hela and HEK with 0.25 % trypsin–0.05 mM EDTA; and U87 with 0.25 % trypsin–0.8 mM EDTA, according to the distributor’s instructions) and diluted at a density of 5×10⁵ cells/ml in MTT assays. Stock cultures were passaged every third day after harvesting the cells with 0.05 % trypsin and seeding 8×10⁵ cells/cm² in tissue culture flasks to maintain the cells in the exponential phase. All experiments were carried out in exponentially growing cells. The cell suspension was seeded into 96-well plates (Corning, USA) at 100 µl/well, and incubated for approximately 24h before tests in order to reach confluency. Before the cells were seeded into 96-well plates, the plates were treated with 0.01 % poly-D-lysine solution (Sigma-Aldrich, Germany).

In vitro cell growth inhibition assay (MTT assay)
The cells were seeded in 96-well plates at a concentration of 1 × 10⁴ cells/well in 200 µL of complete media and incubated for 24, 48 and 72 h at 37 °C in 5 % CO₂ atmosphere to allow for cell adhesion. Stock solutions (2 mg/ml) of the compounds made in PBS were filter-sterilized, then further diluted up to 0.45 µg/ml incomplete media for treatment against 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 solutions (5 mg mL⁻¹ dl H₂O) 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 precipitated 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 IC₅₀ which is directly calculated from % viability (directly proportional to metabolic active cell number). Percentage (%) viability was calculated as: % viability = OD in sample well/OD in control well × 100.

Cell survival assay
After harvesting with 0.05 % trypsin, 150–400 (depending on the treatment) cells were plated 10–14 h before treating with varying concentrations of drugs in DMEM at 37 °C. Cultured cells were treated with doses (1, 10, 50 and 100 µg/ml) of complexes. After the treatment, cells were incubated in dark under humidified, 5 % CO₂ atmosphere at 37 °C for 24 h to allow next growth, then washed to remove the drug and cell survival was determined by counting of cells³⁷. Survival graphs were constructed from three independent experiments by least-squares regression fitting averaged survival levels.

Micronuclei formation
Air-dried slides containing acetic acid–methanol (1:3 v/v) fixed cells were stained with Hoechst-33342 [10 g/ml in PBS (0.1 M), disodium phosphate (0.45 M) buffer containing 0.05 % Tween-20 detergent]³⁸. Slides were examined under the fluorescence microscope using an UV excitation filter. Fluorescent nuclei were visualized using a blue emission filter.
Cells containing micronuclei were counted from >1000 cells by employing the criteria of Countrymen and Heddle\textsuperscript{39}. The fraction of cells containing micronuclei, called the M-fraction (%) or MN frequency was calculated as follows: M-fraction (%) = \( \frac{N_m}{N_t} \times 100 \), where \( N_m \) is the number of cells with micronuclei and \( N_t \) is the total number of cells analyzed. Since micronuclei formation is linked to cell proliferation, the micronuclei frequencies were normalized with respect to the cell numbers.

Detection of apoptotic cells

Morphologically, marked condensation and margination of chromatin, fragmentation of nuclei and cell shrinkage characterize apoptotic cells and a good correlation between these morphological changes and DNA fragmentation (ladder) as hallmarks has been demonstrated\textsuperscript{38}. The percentage of cells undergoing apoptosis was determined microscopically using Hoechst-33342 labeled cells. At least 1000 cells were counted and the percentage of apoptotic cells was determined from slides prepared as described for the micronuclei formation.

Results and Discussion

Ligand design and synthesis

A new set of amide-amine based bidentate ligands; HL\textsuperscript{1} and HL\textsuperscript{2} were designed to offer coordinations via O\textsubscript{amide} and N\textsubscript{amine} atoms in their protonated form. This coordination mode may results in the formation of bis–ligated metal complexes. Both ligands were synthesized in two steps starting from the corresponding primary amine (Scheme 1). In the first step, the primary amine is converted into the respective chloroacetamide which was reacted with the diethyl amine in second step to afford the desired ligand. Both ligands are thoroughly characterized using various spectroscopic measurements and provide satisfactory microanalytical results.

Synthesis and characterization of copper(II) complexes

Copper complexes (1) and (2) were synthesized by the reaction of ligand HL\textsuperscript{1} or HL\textsuperscript{2}, respectively, with [Cu(H\textsubscript{2}O)\textsubscript{6}][ClO\textsubscript{4}\textsubscript{2}] in CH\textsubscript{3}OH (Scheme 2). Both complexes were isolated as dark blue colored crystalline products in good recrystallized yield. The FTIR spectra\textsuperscript{40} of complexes (1) and (2) show absorptions at 3294 and 3296 cm\textsuperscript{-1}, respectively, due to the presence of N-H stretch and thus indicate the protonated form of the amide group in ligand. The amide \( \nu_{\text{C}=\text{O}} \) stretch was observed at 1632 and 1625 cm\textsuperscript{-1} for complexes (1) and (2), respectively. These stretches are red-shifted by 30 – 40 cm\textsuperscript{-1} compared to free ligand and indicate the involvement of O\textsubscript{amide} in the bonding. The split stretches for ClO\textsubscript{4}\textsuperscript{-} were noticed in the region of 1127–1055 cm\textsuperscript{-1} and indicate the presence of coordinated as well as ionic perchlorate ions\textsuperscript{40}. The solution conductivity\textsuperscript{41} data confirms the 1:2 electrolytic nature for both the complexes, whereas the elemental analysis authenticates the purity of the bulk sample.

Crystal structure studies

One of the representative complexes, compound (1) was characterized by the single crystal diffraction studies. The molecular structure of complex (1) is shown in Fig. 1 while the important crystallographic and structural details are summarized in Tables 1 and 2, respectively. The crystal structure shows that two bidentate ligands are coordinated to the Cu(II) ion through the O\textsubscript{amide} and N\textsubscript{amine} atoms (Fig. 1). Two protonated ligands are arranged in a trans fashion around the copper ion where O–Cu–O and N–Cu–N angles exceeds 175°. The 5\textsuperscript{th} coordination comes from...
the weakly coordinated perchlorate ion (Cu–O3: 2.452 Å). The geometry around the copper(II) ion is best described as square–pyramidal with a very small trigonal–bipyramidal distortion parameter ($\tau$\textsuperscript{42} of 0.0038 [$\tau = (\beta-\alpha)/60$, with $\alpha$ and $\beta$ being the two largest coordination angles, 175.54° and 175.31°, respectively]. In a perfect square-pyramidal geometry $\tau$ equals 0, while it is 1 in a perfect trigonal–bipyramidal geometry\textsuperscript{42}. The copper ion is almost within the $N_2O_2$ basal plane with a small deviation of 0.076 Å towards the axially coordinated $ClO_4^-$ ion. The average Cu – O\textsubscript{amide} and Cu – N\textsubscript{amine} bond distances are 1.936 Å and 2.031 Å, respectively; while the average O\textsubscript{amide} – Cu – N\textsubscript{amine} bond angle is ca. 84° (Table 2). For complex (I), the observed Cu–O\textsubscript{amide} and Cu–N\textsubscript{amine} bond distances are comparatively on the lower side than other structurally characterized complexes\textsuperscript{27–29,c,28e,43}. Two ligands create a $N_2O_2$ basal plane around the metal center through two five-membered chelate ring via O\textsubscript{amide} and N\textsubscript{amine} coordinations. Two five-membered chelate rings make an angle of 24.71° with each other and explain the relative twisting of the basal plane around the copper ion. The atoms involved in the constitution of five-membered chelate rings are not co-planar. In fact, a higher displacement from the mean plane was observed for the carbon atoms (0.44 – 0.81 Å) than the heteroatoms. The second perchlorate ion remains uncoordinated, however, was found to engage in several weak hydrogen–bonding interactions (see below).

Careful examination of weak interactions for complex (I) shows an interesting packing behaviour (Fig. 2). The asymmetric unit cell contains four individual molecules along with coordinated and uncoordinated perchlorate ions. The cations and anions were found to connect to each other via C–H···O H-bonding interactions. The uncoordinated perchlorate ion acts as a bridging unit between two cationic molecules and form H-bonds with amidic

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**Table 2**—Selected bond distances (Å) and angles (°) for $[Cu(\text{HL}_2)^2(ClO_4)_2]$ (I)

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<tr>
<td>O2 – Cu – N4</td>
<td>84.15 (14)</td>
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<tr>
<td>O1 – Cu – N4</td>
<td>97.25 (15)</td>
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<tr>
<td>N2 – Cu – N4</td>
<td>175.54 (17)</td>
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Fig. 1—Molecular structure of complex (I). Thermal ellipsoids are drawn at 50% probability level, hydrogen atoms are omitted for clarity. The coordinated and un-coordinated $ClO_4^-$ ions are shown in two different colors for clarity.

Fig. 2—Packing diagram and weak interactions for complex (I) with partial numbering scheme. The hydrogen bonds are shown by the orange colored dots between amidic–NH or phenyl–CH groups and $ClO_4^-$ ions. Only hydrogen atoms involved in hydrogen bonding are shown for clarity.
protons (N1-H and N3-H) from two different molecules to afford a one-dimensional linear chain. The O7ClO4−···N1 and O7ClO4−···N3 heteroatom distances were found to be 2.867 Å and 2.987 Å, respectively. One such chain is further connected to the adjacent chain through the coordinated perchlorate ion via C-H···O bond between O5 atom of ClO4− and phenyl C5-H proton to result in a two-dimensional network. (Fig. 2, view along a – axis). The O5····C5 heteroatom distance was found to be 3.630 Å.

Absorption spectral studies

The solid state absorption spectra of both complexes were recorded in paraffin oil. The features are broad; however, meaningful information can be obtained. For complex (1), a broad band was observed at 540 nm with a weak shoulder at 660 nm indicating a square-pyramidal geometry. This result is corroborated by the observation of coordinated ClO4− anion in the crystal structure of complex (1) (Fig. 1) as well as the IR spectra. The other features were observed at 340 and 250 nm. Similarly, for complex (2), broad features were obtained at 570, 680, 725, and 775 nm. In the MeCN solution, both complexes exhibit a single absorption at 620 nm which could be assigned as the d-d transition considering its low extinction coefficient value. Another band at 303 nm could be assigned as ligand-to-copper charge transfer. The band at 240 nm may be assigned as the intra-ligand charge transfer. The difference in the solution state absorption spectra to that of solid state, can be explained by a change in geometry around the copper(II) center. Results suggest that the square-pyramidal geometry in the solid state changes to tetragonal in solution with possible dissociation of ClO4− ion and further interaction from the solvent molecules.

EPR spectral studies

To extract more information about the geometry around the Cu(II) center, the X-band EPR spectra were recorded as microcrystalline solid as well as MeCN solution at room temperature. For complex (1), the solid state spectrum shows a broad strong feature at $g_z = 2.067$ with a weak shoulder at 2.120 that has been assigned as $g_{||}$. However, complex (2) shows distinct $g$ features (2.244) with the observation of $A_{||}$ (200 G) and a strong $g_z$ signal at 2.054. The observed $g$ values are in the range of tetragonal copper(II) complexes with loosely bound axial ligand. Interestingly, the crystal structure of complex (1) does not show perchlorate ion as the fifth ligand at the axial position (vide supra). It is well documented that tetragonal copper(II) complexes show four-line pattern which is characteristic of hyperfine coupling of the electron spin with the nuclear spin of copper ion ($I = 3/2$). The $g_{iso}$ were observed at 2.14 and 2.16 with the $A(Cu)_{iso}$ values of ~70 G and 65 G, for complexes (1) and (2), respectively.

To investigate spin state, the magnetic susceptibility measurements were performed at 298 K in MeCN solution following Evans’ NMR spectral method. The experimental value of 1.85 for complex (1); and 1.94 BM for complex (2) clearly indicates the $S = 1/2$ ground state and also excludes any possible inter-molecular interaction. Moreover, the observed magnetic moment corroborates the EPR studies for both complexes, which supports the presence of the unpaired electron in the $d_{x^2-y^2}$ orbital.

Inhibitory effects of complexes (1) and (2) on the proliferation of U87 brain cancer, HeLa and normal HEK cells

The MTT cell proliferation assay has been widely accepted as a reliable way to measure the cell proliferation rate, and conversely when metabolic events lead to apoptosis or necrosis. The data obtained by the MTT assay show that both complexes have inhibitory effects on the growth of U87 brain and HeLa cervical cancer cells in dose-dependent manner. Both complexes effectively inhibited the U87 cell growth, with their IC50 values ranging from 17 to 62.5 µg/ml (Table 3). Notably, complex (2) could inhibit the cell growth at lower concentration range of 17 – 31.25 µg/ml after 48 and 72 h of the treatment on U87 cells. On HeLa cells, the cytotoxicity of both

<table>
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<th>Complex</th>
<th>U87</th>
<th>HeLa</th>
<th>HEK</th>
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<tr>
<td>(1)</td>
<td>61(±1.4)</td>
<td>30(±0.6)</td>
<td>30(±0.6)</td>
</tr>
<tr>
<td>(2)</td>
<td>62.5</td>
<td>34(±5.4, ±1)</td>
<td>17(±2.5)</td>
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*Mean (±SD), n = 3.*
complexes decreases in a time dependent manner. For both complexes, the IC$_{50}$ values were found to be 31.25 µg/ml at 24 h which further increased to 65 µg/ml after 72 h of treatment. Both complexes were found to be less cytotoxic on the normal HEK cells when compared with U87 and HeLa cells (Table 3). The data suggest that the complexes are less cytotoxic on normal HEK cells at moderate concentrations (250 – 0.45 µg/ml) with their IC$_{50}$ values in the range of 125–250 µg/ml. It may be noted that the typical IC$_{50}$ values for the commercial drug cis-platin falls in the range of 2.6–20 µM on U87 cells$^{47}$; 1.34–2.20 µM on HeLa cells$^{48}$; and 7–14 µM on HEK cells$^{49}$. The importance of such work lies in the possibility that the next generation metal complexes might be more efficacious as 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 anticancer agents for the therapeutic use.

Inhibitory effects of complexes (1) and (2) on the survival of U87 and HeLa cells.

For survival studies, cells were incubated with complexes (1) and (2) continuously and then washed to remove the metal complexes. The cell survival was determined at the complex concentration of 1, 10, 50 and 100 µg/ml. At these concentrations; complex (2) was able to kill 12 %, 32 %, 49 % and 73 % of the cells, respectively. On the other hand, complex (1) at identical concentrations was able to kill 8 %, 27 %, 48 % and 71 % cells (Fig. 3). At low concentration of 10 µg/ml, complex (2) showed better toxicity (32 %) than complex (1) (25 %) on the U87 cells. On HeLa cells at concentrations of 1, 10, 50 and 100 µg/ml; complex (2) killed 26 %, 45 %, 61 % and 89 % of the cells; whereas complex (1) was able to kill 24 %, 43 %, 58 %, 85 % cells, respectively (Fig. 3).

Cytogenetic damage

Mitotic death (linked to cytogenetic damage) and inter-phase death (apoptosis) together account for the cytotoxicity of many physicochemical agents, although the relative contributions of the two death processes vary among the type of damaging agent$^{50}$. To investigate the modifications of the cytogenetic damage by metal complexes, we studied the treatment-induced micronuclei formation in gliomas and cervical cancer cells. Since cell proliferation influences treatment induced micronuclei expression, the data from kinetic studies up to 24 h post treatment were analyzed. Complexes (1) and (2) show significant micronuclei frequency on HeLa cells than...
on U87 cells. The frequency of cells with micronuclei was in the range of 15–30% on both cell lines at 31.25 µg/ml. Micronuclei frequency of complex (2) was found to be 3–4 times more when compared with untreated control groups on both cell lines. Interestingly, more than 22–30% cells were found with one or more micronuclei in these treated groups at 31.25 µg/ml concentrations on HeLa cells (Fig. 4).

**Complexes induced apoptosis**

The apoptotic mode of cell death in both cell lines was confirmed by microscopy. The cell morphological changes were characterized by typical chromatin condensation and nuclear fragmentation with disruption of plasma membrane integrity shown that the metal complexes induced a significant level of apoptosis in gliomas and HeLa cells. In particular, complex (2) showed significant apoptosis frequency. Apoptotic frequency of complex (2) treated cells is 9–14% which is four–fold times that of untreated control cells on both cell lines (Fig. 4). On both cell lines, complex (2) showed significant increase in both apoptotic frequency and MNi frequency than complex (1).

**Conclusions**

The present work demonstrates the synthesis and characterization of two copper(II) complexes with amide-amine based bidentate ligands. The ligands coordinate the metal ion through Oamide and Namine centers maintaining a N₂O₂ basal plane. The crystal structure of complex (1) shows square-pyramidal geometry around the metalion whereas two ligands coordinate the Cu(II) ion in the basal plane whereas the axial position is occupied by the ClO₄⁻ ion. In both complexes, the Cu(II) ion is in magnetically dilute environment as suggested by the EPR spectra and magnetic measurements. Both copper complexes were then tested for the anticancer studies. The results show that the treatments with complexes sensitize U87 and HeLa cells time dependently by increasing both mitotic and apoptosis death. These conditions favor chromosome condensation expressed as enhanced chromosomal aberrations manifesting in mitotic death. In HeLa cell lines, the enhanced cell death due to the copper complexes was also accompanied by a significant increase in micronuclei formation. The copper complexes are proposed to induce a higher rate of faulty mitosis in damaged cells, which could partly contribute to the enhanced micronuclei observed. Further studies on mechanism by which these complexes induce micronuclei formation and apoptosis in U87 gliomas, HeLa and other cancer cells are ongoing and may help in designing better and efficient metal-based drugs. In addition, the better cytotoxicity of complex (2) (with ethylbenzene substituent) than complex (1) (with benzyl substituent) against both HeLa and U87 cells depicts the importance of ligand design in medicinal inorganic chemistry. Such findings contribute to the establishment of a general rule that the biological activity and mechanism of action of metal complexes can be fine-tuned by an appropriate choice of the metal and the coordinated ligand.

**Supplementary Data**

CCDC 798084 contains the crystallographic data for the complex (I) reported herein. These data may be obtained free of charges from the Cambridge Crystallographic Data Center, 12, Union Road, Cambridge CB2 1EZ UK via www.ccdc.cam.ac.uk/datarequest.cif.

**Acknowledgement**

RG thanks University of Delhi, Delhi, for financial support under the scheme to strengthen R&D, IIT Kanpur, Kanpur, for crystallographic data collection and CIF-USIC center of this university for instrumental facilities. APS thanks CSIR, New Delhi, for SRF fellowship.

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