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Preliminary communication

Synthesis, characterization and biological properties of a novel copper complex

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Abstract

The study of copper complex in relation to cancer is important in many ways. A novel copper complex has been synthesized with non toxic ligand, viz. potassium salt of *N*-(2-hydroxy acetophenone) glycinate (NHAG). The structure of the complex has been determined by spectroscopic means. Toxicity and antitumor property of the complex has been studied in vivo. Though the complex is toxic at higher doses, lower non toxic doses of the complex deplete glutathione (GSH). GSH depleting property of the complex may be utilized to sensitize drug resistant cells where resistance is due to elevated level of GSH.

Keywords: Coordination; Copper; Glutathione; *N*-(2-hydroxy acetophenone) glycinate

1. Introduction

Coordination of organic compound with metal i.e., chelation causes drastic change in the biological property of the ligand and also the metal moiety [1]. It has been reported that chelation is the cause and cure of many diseases including cancer. The metal copper [Cu(II)] is also involved in the causation and cure of cancer [2–7]. Copper administration suppresses rat hepatoma induced by chemical carcinogen [8]. Copper(II) complexes cause tumor cells to redifferentiate into normal cells [9]. Although physiological role of copper is controversial, it is proved that copper is an essential component of several endogenous antioxidant enzymes [10].

Coordination behavior of sodium or potassium salt of *N*-(2-hydroxy acetophenone) glycine (NHAG) towards organotin moiety has recently been reported [11]. Antitumor property of the ligand [NHAG] and some of its organotin complexes has been studied [12]. The non-toxic nature, water solubility and strong coordination behavior of NHAG deserve attention [11,12].

The present work describes the synthesis of a novel copper complex with potassium salt of NHAG; the structure of the complex has been determined by spectroscopic studies. Antitumor property, toxicity and glutathione (GSH) depletion property in vivo of the complex has also been reported.

2. Chemistry

2.1. Materials and methods

2.1.1. Chemicals

N-(2-hydroxy) acetophenone, copper sulphate, glycine, DMSO was purchased from Aldrich, NY. Ethacrynic acid (EA), buthionine sulphoxamine (BSO), verapamil, dithio nitrobenzene (DTNB) were purchased from Sigma Chemical Compamy, St. Louis, MO. Other chemicals used were of highest purity available.

Abbreviations: NHAG, *N*-(2-hydroxy acetophenone) glycine; CuNG, copper *N*-(2-hydroxy acetophenone) glycinate; BSO, buthionine sulphoxamine; EA, ethacrynic acid; DTNB, 5,5′-dithio bis (2-nitrobenzoic acid); DMSO, dimethyl sulphoxide; EAC, Ehrlich ascites carcinoma.

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2.2. Synthesis of the ligand

The ligand, potassium *N*-(2-hydroxy acetophenone) glycinate was prepared according to the reported methods [11]. In brief, a cold aqueous solution of KOH (1.03 g, in 12 mL) was mixed with cold aqueous solution of glycine (1.38 g in 12 mL) and held at 15–20 °C in an ice bath with continuous stirring. An ethanolic solution of 2-(hydroxy) acetophenone (2.5 g in 25 mL) was added dropwise. Deep yellow color was developed and stirring was continued for 1 h followed by 5 h at room temperature. The solvent was removed by rotary evaporator. The yellow mass was washed with pet–ether and precipitated with methanol–diethyl ether mixture. The crude product was recrystallised from methanol to yield potassium *N*-(2-hydroxyacetophenone) glycinate. Yield 75%, m.p. 258–260 °C.

2.3. Synthesis of the copper complex

CuNG was synthesized from the ligand, potassium *N*-(2-hydroxy acetophenone) glycinate by its reaction with copper sulphate; in brief, 0.68 g $CuSO_4$, $5H_2O$ was dissolved in 5 mL deionised water. 0.785 g potassium *N*-(2-hydroxy acetophenone) glycinate was dissolved in 25 mL ethanol. The solution of *N*-(2-hydroxy acetophenone) glycinate (yellow color) was slowly added to $CuSO_4$ solution (blue color) at room temperature with continuous stirring by a magnetic stirrer. The mixture was further stirred in the magnetic stirrer for 1 h at 45–50 °C. The color of the mixture changed to deep green. The mixture was cooled to room temperature and the green precipitate was separated by filtration. The compound was dried and recrystallised from DMSO. Yield 40%, m.p.242°C. Anal. Calc. $C_{10}H_{15}O_6NCu$: C, 39.0; H, 4.87; N, 4.54; Cu, 20.45. Found: C, 40.57; H, 5.04; N, 4.65; Cu, 21.24%.

3. Pharmacology

3.1. Biological materials

All animals were collected from our animal colony.

EAC was maintained as an ascitic tumor in male Swiss albino mice weighing 18–20 g (6–8 weeks old).

3.2. Study of in vivo toxicity

3.2.1. Effect of CuNG on blood

CuNG (1 mg) was dissolved in 1 mL DMSO. The solution (0.1 mL) was injected to male Swiss albino mice (i.e. 5mg kg^{-1}). Blood was collected from normal and also from treated mice after different time interval of CuNG injection intraperitoneally.

Blood was obtained via closed cardiac puncture by means of a 22-guage hypodermic needle and a sub-xiphoid approach. Blood from each group (CuNG treated and untreated) was pooled into separate glass tubes and treated with anticoagulant (heparin).

Normal and differential blood count was done for treated and normal mice. The experiment was repeated for four times.

3.2.2. Effect of CuNG on spleen

CuNG (5 mg kg^{-1}) in DMSO was injected into male Swiss mice.

3.3. Preparation of spleen cell suspension

Normal and CuNG treated male Swiss mice were anaesthetized and 70% alcohol was sprayed on abdominal region. Spleen was removed aseptically and small amount of PBS was injected to it; Spleen was rubbed against the fine wire mesh of the tissue grinder. The cell suspension formed is spinned at 1000–1500 rpm for 5–10 min. The supernatant was discarded and the cells were washed by spinning in PBS twice at room temperature.

Cell viability was tested by trypan blue and cells were counted in a phase contrast microscope. The experiment was repeated for four times.

3.3.1. Effect of CuNG on bone marrow

CuNG (5 mg kg^{-1}) in DMSO was injected to male Swiss mice.

3.4. Separation of bone marrow cells

Normal and CuNG treated mice were anaesthetized and the femur bone was cut with the help of a vertebrate scissor. Bone marrow was flushed with 0.56% KCl solution and centrifuged at 3000 rpm for 15 min at 37 °C.

Cells were counted under microscope for treated and untreated animals. The experiment was repeated for four times.

3.5. Study of antitumor property of CuNG in vivo

Fifty-five male Swiss albino mice 6 weeks of age were divided into six groups; one control group with five mice and five drug treated groups with 10 mice in each group. EAC cells (1×10^6) were injected i.p. to all mice on day 1. On day 2, various doses of the ligand (NHAG) dissolved in deionised and autoclaved water were injected i.p. to mice of various groups. No drug was administered to the control group. Life monitoring was restricted to daily body weight measurement, recording time of death. Animals were observed for a period of 60 days. Cell yield, ascites volume, packed cell volume,

mean survival time (MST) and change in life span of the treated mice in comparison to control (T/C value) were recorded. The experiment was repeated for four times.

3.5.1. Effect of CuNG on survival of animals

Various doses of CuNG dissolved in DMSO were injected ip to male Swiss mice. Animals were observed for a period of 72 h. The average value of the animals living (percentage) of three independent experiments with respect to the doses of CuNG was plotted (Fig. 2). The amount of CuNG required to kill 50% of the animals (IC_{50} value) in vivo was determined from the plot.

3.6. GSH-depleting properties of CuNG in vivo

Cells (1×10^6) were injected i.p. into male Swiss mice 6 weeks of age. On the second day, 1 mg kg^{-1} CuNG was injected to mice. On the 12th day animals were anesthetized, killed and EAC cells were collected. Cells (1×10^6) were washed in PBS twice. Cells were homogenized and divided into two equal amounts for measuring GSH and protein.

EA, BSO, verapamil were also injected in the similar manner and cells were collected.

Normal cells were taken from EAC bearing mice 8 weeks of age not undergone any drug treatment. Experiment for each drug treated and control group were repeated for four times.

GSH was measured by the method of Sedlack and Lindsay [13] and protein was measured by Lowry [14].

4. Results and discussions

UV spectrum for the complex and the ligand was taken in methanol.

UV bands for the ligand λ_{max} (methanol): 271, 350, 411.

UV bands for the complex λ_{max} (methanol): 271, 337, 380.

The change in the UV peak from 350 in the ligand to 337 in the complex indicates π–π* transition. The shift of the peak from 411 in the ligand to 380 in the complex also indicates π–π* transition.

Important infrared (IR) bands for the ligand appear at: 3410–3360, 1689, 1619, 1524, 1466, 1421, 1395, 1318, 1269, 1205, 1163, 969, 931, 752, 730.

Important IR bands for the complex appear at: 3407–2700, 2340, 1630, 1604, 1541, 1465, 1444, 1364, 1309,1237, 1160, 1088, 1071, 1035, 965, 941, 861, 785, 762, 722, 622, 595, 537, 526, 453.

The IR spectrum of the ligand shows broad band at 3360–3410 cm^{-1} which may be due to νOH. The free νOH is generally observed between 3400 and 3500 cm^{-1}. The observed low value is due to intramolecular H-bonding between H and nitrogen [11]. In the copper complex the band shifted to 2700–3407 cm^{-1}. The alkyl groups CH_2, CH_3 show characteristic deforming bands at 1466–1395 cm^{-1} and the rocking modes at ~730 cm^{-1} in the ligand. The rocking mode of the ligand shifts to 762 cm^{-1} in the complex. The band at 1604 cm^{-1} is assigned due to νCN in the complex (in the ligand the band appears at 1619). The sharp band at 1395 cm^{-1} in the ligand may be assigned due to νOCO symmetric that has been shifted to 1364 cm^{-1} in the complex indicating coordination with the Cu-atom through the COO^- group of the ligand. The band at 1269 cm^{-1} in the ligand may be assigned due to νPh(CO) which has been shifted to 1237 cm^{-1} in the complex indicating coordination of the phenolic oxygen to the Cu-atom. The band at 1689 cm^{-1} in the ligand may be assigned due to a ν(OCO) asymmetric vibration and in complex this vibration appears as broad band at 1630 cm^{-1} [12].

Thus, the IR spectra results provide strong evidence for the complexation of the potentially multidentate ligand.

Proton NMR peak of the ligand in D_2O appears at 7.38–7.51 (s, 5H) and 6.59–7.6 (s, 3H) for aromatic protons. CH_2 protons appear at 4.09 (1H, m). CH_3 protons appear at 2.26–2.29 (4H, m).

Proton NMR peak of CuNG in DMSO-d_6 appear at 5.59 (S) for aromatic protons. CH_2 protons appear 3.40 (B). CH_3 protons appear at 2.51–2.79 (m, 4H). In NMR spectra we observe a shift of electron density from the ligand to metal moiety. Aromatic protons shifts from the range of 7.38–7.51 in the ligand to 5.99 in the complex. Methelene protons shift from 4.09 in the ligand to 3.40 in the complex. The shielding of the aromatic and methylene protons indicate the formation of the complex. However, the methyl protons in the ligand shifted to higher ppm; from ligand 2.26–2.29 to 2.51–2.79 in the complex. Probably, methyl protons were not pulled from ligand to metal moiety.

Mass spectral data is presented in Fig. 1. The formation of molecular ion peaks indicates that the structure of the complex is **A** in Fig. 1.

Electron paramagnetic resonance (EPR) spectrum of the complex has been recorded in the polycrystalline state at room temperature. The spectrum shows the value of $g_\perp = 2.06$ and $g_{||} = 2.20$. The value of $g_{||}$ lower than 2.3 indicates a covalent metal–ligand environment [15]. The value of $g_{||} < 2.3$ in the present Cu-complex gives a clear indication of covalent character of the metal–ligand bond and delocalisation of the unpaired electron into the ligand. The trend of $g_{||} > g_\perp > 2.0023$ describes the axial symmetry with the unpaired electron residing in the $d_{x^2-y^2}$ orbital [16].

Magnetic susceptibility study of the present Cu-complex gives a magnetic moment value (μ_{eff}) of 1.98 BM at room temperature. $\mu_{eff} = 1.98$ BM indicates one

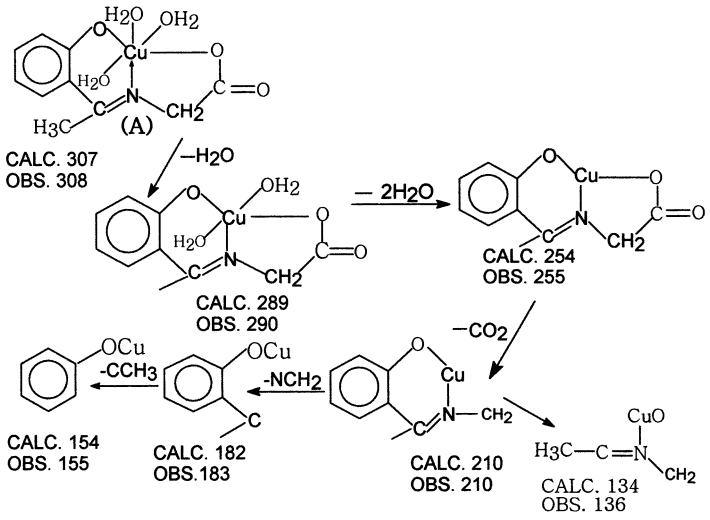

Fig. 1. Mass fragmentation of CuNG.

electron paramagnetism. This value is higher than the spin-only value of 1.73 BM for one unpaired electron. The higher value of the magnetic moment indicates that CuNG is monomeric in nature and there is no metal–metal interaction along the axial position in the complex [17].

Study of hematological toxicity in vivo is presented in Table 1.

The ligand is nontoxic up to 100 mg kg^{-1}. There is no change in hematological parameters at a dose of 50 and 100 mg kg^{-1} of the ligand. The effect was studied at various time intervals like 1, 10 and 25 days of the ligand. CuNG is toxic at a dose of 25 mg kg^{-1}. Lower doses like 6 or 10 mg kg^{-1} do not show any toxic effect. Slight toxicity is shown at 12 mg kg^{-1}. The results presented in Table 1 are based on four independent experiments.

The effect of the complex on spleen and bone marrow is presented in Table 2. The ligand is nontoxic up to 100 mg kg^{-1}. There is no change in spleen or bone marrow cells at a dose of 50 and 100 mg kg^{-1} of the ligand. CuNG is toxic at a dose of 25 mg kg^{-1}. Lower doses like 6 or 10 mg kg^{-1} do not show any toxic effect; 12 mg kg^{-1} shows slight toxicity.

Toxicity of CuNG in vivo is presented in Fig. 2. CuNG at a dose of 35 mg kg^{-1} kills 50% of the animals

Table 2
Effect of CuNG on spleen and bone marrow of male Swiss albino mice

Drug	Dose (mg kg^{-1})	Spleen cell ($\times 10^3$)	Bone marrow cell ($\times 10^3$)
		10D	10D
–	–	4.7±0.82	1.27±0.06
Logan	50	4.65±1.82	1.2±0.82
Ligand	100	4.45±0.41	1.3±0.82
CuNG	6	4.65±0.45	1.01±0.13
CuNG	10	4.61±0.31	0.97±0.08
CuNG	12	3.95±1.15	0.7±0.08
CuNG	25	3.17±0.03	0.65±0.04

The data are means±S.D. of four independent experiments. CuNG at a dose of 10 mg kg^{-1} is significantly non toxic to spleen and bone marrow when compared with untreated control ($p < 0.001$).

Table 1
Effect of CuNG on hematological parameters of male albino Swiss mice

Drug	Dose (mg kg^{-1})	Haemoglobin (Hb) (gm/dl)			WBC ($\times 10^3$)	RBC ($\times 10^6$)	Lymphocyte (%)	Neutrophil (%)
		1D	10D	25D	10D	10D	10D	10D
–	–	11.77±0.31	11.89±0.35	11.91±0.41	2.9±0.08	5.6±0.21	59.2±0.83	36.07±0.33
Ligand	50	11.57±0.49	11.62±0.31	11.81±0.37	2.93±0.12	5.87±0.25	52.03±053	42.07±1.41
Ligand	100	11.83±0.29	11.59±0.14	11.91±0.41	2.84±0.21	5.77±0.13	51.67±1.83	40.07±3.33
CuNG	6	11.9±0.96	11.38±0.28	11.93±0.31	2.89±0.17	5.6±0.08	59.0±2.83	41.07±0.8
CuNG	10	11.71±0.32	11.28±0.13	11.6±0.51	2.79±0.13	5.5±0.11	58.6±2.1	40.10±0.72
CuNG	12	11.9±0.96	10.92±0.31	10.73±0.47	2.36±0.25	3.9±0.21	57.2±2.83	36.87±1.66
CuNG	25	8.3±0.22	8.43±0.35	8.41±0.31	1.25±0.04	2.6±0.08	51.53±1.01	28.02±1.67

The data are means±S.D. of four independent experiments. CuNG at a dose of 10 mg kg^{-1} has no toxic effect on hematological parameters ($p < 0.001$) when compared with untreated control.

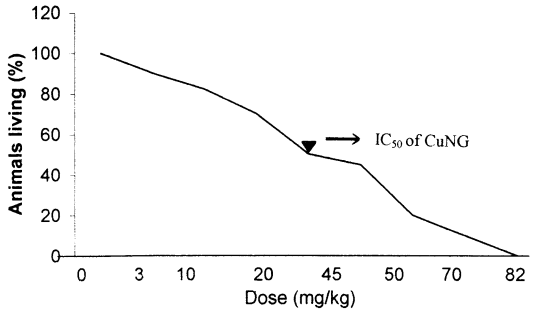

Fig. 2. In vivo toxicity of CuNG. The plot is drawn based upon the average value of three independent experiments of animals living (percentage) with respect to dose of CuNG. IC_{50} value of CuNG is 35 mg kg^{-1}.

(IC_{50} value). The graph is based on the average value of three independent experiments.

The antitumor property of CuNG is presented in Table 3. Neither the ligand nor the complex showed any antitumor property in vivo as reflected in T/C value. The ligand is non toxic at 120 mg kg^{-1}. CuNG is quite toxic at a dose of 35 mg kg^{-1}.

Depletion of GSH by CuNG is shown in Table 4. CuNG is stronger GSH depleting agent than the recommended drug EA [18] at a dose of 10 mg kg^{-1}. Most of the GSH depleting agents reported in literature are effective in vitro and are toxic at required doses when applied in vivo [18–20]. Though CuNG is toxic at higher doses, low non-toxic doses like 2–5 mg kg^{-1} may be effective to deplete GSH.

5. Conclusions

CuNG is toxic at higher doses and depletes GSH even in nontoxic doses. It has been reported that a number of drug resistant cells have higher level of GSH compared to drug sensitive cells and modulation of cellular GSH homeostasis sensitizes drug resistant cancer cells to a wide range of chemotherapeutic drugs [21]. The non toxic dose of CuNG may be utilized to deplete GSH in a number of drug resistant cell lines and thereby overcome drug resistance where resistant is due to higher level of GSH. The study of the effect of CuNG on various sensitive and drug resistant cells are in progress.

6. Experimental protocols

6.1. Chemistry

UV–vis spectra was recorded in Shimadzu UV 160 A and in Varian Cary 100 Scan in the range of 800–200 mμ.

IR spectra were recorded in Perkin–Elmer RX 1 FT spectrophotometer in KBR discs in the range 4500–500 cm^{-1}.

Proton NMR spectra was recorded in DMSO-d_6 on a Bruker ACF 300 spectrometer at 300.13 MHz reference to Me_4Si (0.0 ppm).

Mass spectrum was recorded in an AEI MS-30 mass spectrometer.

C, H, N was measured by Perkin–Elmer 2400 Series II CHN analyzer.

Copper was measured by atomic absorption spectrophotometer Varian Spectra 200 FS, hollow cathode lamp, flame type: air acetylene; replicate 3; wavelength 324.8 nm.

EPR spectrum (Fig. 3) was recorded in Varian E 109C at room temperature at field set: 3200G; scan range 4×1 K; receiver gain 4.0×10^3; scan time 4 min; modulation 1.25×10; time constant 0.250; frequency 9.1 GHz, power 30 dB; temp. 295 K; state: microcrystalline.

Magnetic moment was measured in EG & G vibrating sample magnetometer (model 155).

6.2. Biological evaluation

GSH was measured following the method of Sedlack and Lindsay [13]. Briefly, to 2×10^5 cells in 0.2 mL PBS, 4.8 mL EDTA (0.2 M) was added and kept on ice bath for 10 min. Then 4 mL deionised water and 1 mL of 5% trichloroacetic acid (TCA) were added. The mixture was again kept on ice for 10–15 min and then centrifuged at 3000 rpm for 15 min. Two milliliters of supernatant was

Table 3
Effect of CuNG on EAC bearing male Swiss mice (10 days after CuNG injection)

Parameters	Control	Ligand		CuNG (mg kg^{-1})		
		50	100	6	12	35
Cells in whole ascites fluid (10^6)	170±10.2	173±1.7	171±2.49	174.4±1.63	154±3.27	138±1.67
Total volume (mL) (tumor cells+ascites fluid)	7.5±1.48	7.13±0.2	7.77±0.13	7.27±0.13	5.4±0.52	5.37±0.17
Total packed cell volume (mL)	5.3±0.43	5.4±0.16	5.07±0.13	4.93±0.19	4±0.16	3.4±0.16
Mean survival time (MST) (days)	33.5±2.73	36±1.63	34±1.63	30±1.63	23±1.26	17±0.82
T/C (%)	100	107.46	107.46	89.55	69.64	50.75

The data are means±S.D. of four independent experiments. CuNG at non toxic dose (10 mg kg^{-1}) has no antitumor property when compared with untreated control ($p < 0.005$).

Table 4
Depletion of glutathione (GSH) by CuNG

Drugs	Dose (mg kg^{-1})	GSH (ng μg^{-1} of protein)	% of GSH depletion
No drug (control)	–	225±31	0
Ethacrynic acid (EA)	20	59±9	73
Buthionnine sulphoxamine (BSO)	20	33±5	85
Verapamil	15	221±21	1.78
CuNG	2	151.33±5.31	39.5
CuNG	5	102.67±6.38	58.93
CuNG	10	57.67±5.12	76.93

The values are means±S.D. of four independent experiments. The values of GSH are significantly lowered in EA, BSO and CuNG (10 mg kg^{-1}) treated cases when compared with untreated control ($p < 0.001$).

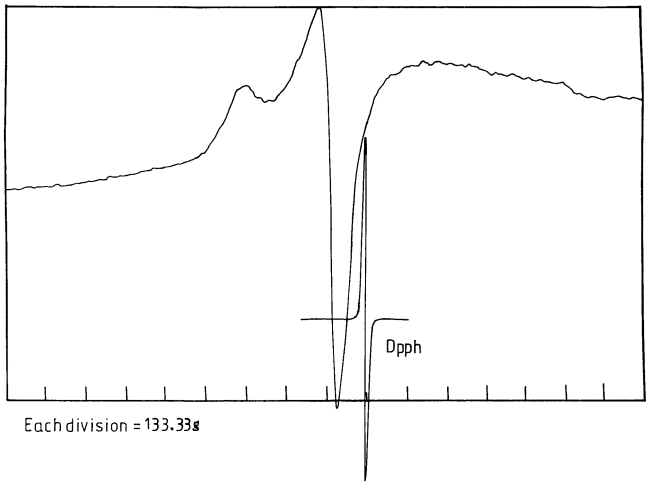

Fig. 3. EPR spectrum of CuNG.

taken and 4 mL of 0.4 M Tris buffer (pH 8.9) was added. 5,5′-dithio-bis-(2-Nitrobenzoic acid) (DTNB) solution (0.1 mL) was added and vortexed thoroughly. Optical density (O.D.) was read (within 2–3 min after addition of 0.1 mL 0.01 M DTNB) at 412 nm against a reagent blank. Appropriate standards were taken and protein was measured according to Lowry et al. [14]. The experiment was repeated for four times.

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