Study the Antitumor Activity of Copper (II) Complex of 4-Azomalononitrile Antipyrine on Mice Induced With Earlich Ascites Carcinoma Cells

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INTRODUCTION
Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion and sometimes metastasize. These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Cancer affects people at all ages with the risk for most types increasing with age [1].

The main goal of cancer therapy is to attain the maximum therapeutic damage of tumor cells with the minimum concentration of the drug. This can be achieved, in principle, via selective antitumor preparations, the cytostatic effects of which would be restricted within tumor tissue. While 100% selectivity may be impractical, achievement of reasonably high selectivity seems to be a feasible aim. The bioenergetic status in tumor was selective and affected by the metal complexes. Minimization of signals of high-energy phosphate was observed after injection of the complexes. An increase in the number of DNA single-strand breaks registered in tumor tissue, supporting the suggestion that the complexes may directly affect DNA; however, the action of these complexes as antitumor agents was found to be dependent on the type of tumor cell line tested [2].

Structure and bond properties of copper (II) complexes are of continuous interest in inorganic chemistry and biochemistry [3]. On the other hand, various ligands phosphonate derivatives are of interest because of their broad spectrum of biological properties. Much attention has been focused on synthesis of phosphonate and phosphate esters of N-heterocyclic systems and their platinum (II) and palladium(II) complexes because of their potential [4] and significant antitumor activity [5].

The present work was undertaken to investigate the antitumor activity of new synthesized of copper (II) complex of 4-azomalononitrile antipyrine which has SOD like activity on tumor development using Ehrlich ascites carcinoma (EAC) implemented in Swiss albino female mice. The results showed that copper complex with ligand has anti-cancer activity in all studied parameters with variable ratios.

ABSTRACT
Copper (II) complex of 4-azomalononitrile antipyrine has been isolated and characterized based on IR spectra, 1H NMR, elemental analyses, molar conductance, electronic spectra and magnetic moment. A diminished amount of antioxidant enzyme coupled with superoxide production in tumor appears to be general characteristic of the tumor cells. This character can be used in cancer treatment. The present study attempted to investigate the effect of copper (II) complex of 4-azomalononitrile antipyrine which has SOD like activity on tumor development using Ehrlich ascites carcinoma (EAC) implemented in Swiss albino female mice. The results showed that copper complex with ligand has anti-cancer activity in all studied parameters with variable ratios.

KEYWORDS
mice earlich ascites carcinoma cells liver and kidney histopathology anti-oxidant enzymes DNA content
Elmer 1430 recording spectrophotometer. The electronic spectra were carried out as solution (10^{-5} M) in DMF using a Perkin-Elmer Lambda4B spectrophotometer. The molar conductivity measurements were made in DMF solution (10^{-3} M) using a Tacussel conductometer type CD6N Magnetic susceptibilities were measured at 27 °C using modified Gouy method with Johnson Mattye balance.

**Synthesis of ligand and copper (II) complex**

4-azomalononitrile antipyrine was prepared by coupling the diazonium salt of 4-aminoadipic acid with malononitrile in sodium acetate solution. The product was recrystallized several times from ethanol. Copper chloride complex was prepared by magnetically stirring 0.001 mole of copper perchlorate salt solution with 0.001 mole of the 4-azomalononitrile antipyrine at 60 °C in certain 50 ml ethanol solution, then added to the mixture of sodium acetate solution for periods of two hours. The resulting solids was filtered, washed several times with ethanol and dried under vacuum over P_{2}O_{5}.

**Tumor cell line**

Ehrlich ascites carcinoma (EAC) cells was donated from Tumor cell line (5x10^{6} cells/ml) from a donor mouse bearing 6-8 days-old ascites tumor, into three mice to ensure that the ascetics fluid will be still propagated and can then be drawn from at least on life mouse.

**Experimental design**

All experiments were carried out on adult female Swiss albino mice, purchased from Theodore Bilhariz Research Institute, Ministry of Scientific Research, Giza, Egypt, with an average body weight of 25-30 gm. Mice were housed at the animal house of Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City. EAC cells were propagated by weekly intraperitoneal injection of 0.2 ml of freshly drawn ascitic fluid diluted in phosphate buffer saline solution contains (5x10^{6} EAC cells/ml) from a donor mouse bearing 6-8 days-old ascetic tumor, into three mice to ensure that the ascetics fluid will be still propagated and can then be drawn from at least on life mouse.

**Induction with Earlish Ascites Carcinoma (EAC) Cells Earlich**

Asbestos Carcinoma (EAC) cells are resuspended in phosphate buffer saline solution at a concentration of 5x10^{6} cell / ml, 0.2 ml of cell suspension containing 1x10^{6} cells, viability ≥ 95 % injected intraperitoneally for 24 hrs before treatment.

**Copper (II) complex treatment**

Predetermined 0.2 ml therapeutic dose containing 10000 µM injected intraperitoneally in Cu(ClO)_{4} treated group in the site of tumor induction. Treatment takes place three times a week for three weeks.

**Free radicals and antioxidant enzymes study**

Malondialdehyde (MDA) Level: measured according to Satoh [6], by Lipid Peroxidation Colorimetric Assay Kit; Oxford Biomedical Research, Inc., Oxford, MI 48371 U.S.A. This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA and HAE at 45 °C. One molecule of either MDA or HAE reacts with 2molecules of Reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm.

**Nitric oxide assay**

Measured according to Schmidt et al. [7] by Colorimetric Non-enzymatic nitric oxide assay kit; Oxford Biomedical Research, Oxford MI 48371, USA, Product catalogue NB 88. This kit allows to measure total nitric oxide (NO) produced in experimental systems following conversion of nitrate to nitrite by metallic cadmium.

**Superoxide dismutase activity assay**

Activity was measured by the Superoxide Dismutase activity assay kit, Catalog Numbers, STA-340, Cell Biolabs, Inc. 10225 Barnes Canyon Road, Suite A103, San Diego, CA 92121. Principle of the test is based on super oxide anions (O2-) are generated by a xanthine / xanthine oxidase (XOD) system, then detected with a chromagen solution. In the presence of SOD, these superoxide anion concentrations are reduced, yielding colorimetric signal.

**Glutathione peroxidase assay**

Activity was measured according to Beulter [8] by the Glutathione Peroxidase assay Kit, catalogue no.703102, Cayman Chemical Company, USA. Glutathione Peroxidase measures GPX activity indirectly by a coupled reaction with glutathione reductase, oxidized glutathione, produced upon reduction of hypoperoxide by GPX, is recycled to its reduced state by glutathione reductase and NADPH. Glutathione was measured by the Glutathione Assay Kit, QuantiChrom TM Bio- assay Systems, 3423 Investment Boulevard, Suite, Hayward, CA 94545, USA. Direct colorimetric determination of reduced glutathione was carried out at 412nm in biological samples. The 5, 5’dithiobis (2-nitrobenzoic acid (DTNB) method combines deproteination and detection (Reagent A) into one reagent. DTNB reacts with reduced glutathione to form a yellow product.

**Liver histopathological study**

Liver tissue samples were fixed in 10% neutral buffered formalin, transferred to 70% -80%-90%-95% and 100% ethanol for dehydration, then the tissues were cleared by the removal of alcohol by replacement with xylene before embedding in paraffin and microtome sectioning. Liver sections were stained with eosin and hematoxlin (H&E), and then mounted in Canada balsam for examination by a light microscope.

**Cellular DNA content study**

The nuclear DNA content of a cell can be quantitatively measured at high speed by Flow Cytometry (FACS Calibur flow cytometer, Becton Dickinson, Heidelberg, Germany) according to Peter [9]. The analysis of the cell cycle measured according to Rafael [10].
RESULTS

\[1\]H NMR and infrared spectra

The \[1\]H NMR spectrum of the 4-azomalononitrile antipyrine ligand is reported (El-Boraey et al. [11]). The reaction of copper chloride with the ligand 1:1 metal complex produce complex of formula [CuL (OH) (ClO\textsubscript{4})]. The complex was stable under ordinary conditions; they were freely soluble in DMF and DMSO. The value of molar conductivities in DMF (10\textsuperscript{3}) solution in which the complex is nonelectrolyte [12]. The study and comparison of the IR spectra of the ligand and its copper (II) complex imply that the ligand is bidentate in nature with carbonyl oxygen and azomethine nitrogen, as two coordination sites.

The infrared spectrum of the ligand shows four bands at 3190, 2210, 1630 and 1590 cm\textsuperscript{-1} assigned to v(N-H), v(C=N), (C=O) and v(C≡N) respectively. The infrared spectra of copper (II) complex show a decrease in energy of v(C≡O), v(C≡N) on complexation and appear at 1573 cm\textsuperscript{-1} and 1565 cm\textsuperscript{-1} which assigned to v(C≡O) and v(C≡N) respectively. The IR spectra of copper (II) complex shows that the bands corresponding to v(N-H) and v(C≡N) at the same frequencies compared to free ligand, indicating that the N-H and (C≡N) groups do not participate in coordination.

However some new bands with medium to weak intensities appear in the 455 cm\textsuperscript{-1}, 380 cm\textsuperscript{-1} and 325 cm\textsuperscript{-1} assigned to v(M-O), v(M-N) and v(M-Cl) [13, 14, 15]. The IR spectra of copper (II) complex exhibits a very strong split band (vs) at 1110 cm\textsuperscript{-1} and 1070 cm\textsuperscript{-1} and strong band at 625 cm\textsuperscript{-1} (va), which are indicative of monodentate coordinate perchlorate [16]. The IR spectra of hydroxo- complex shows band at 3380 cm\textsuperscript{-1}, assigned to v(M-OH) [17].

Magnetic and electronic spectral studies

The copper (II) complex exhibits magnetic moment value close to spin only value (1.73 B.M.). Excluding the possibility of spin- spin interaction between the copper (II) centers. The electronic spectra of the copper (II) complex show bands at 1700 cm\textsuperscript{-1} and 14000 cm\textsuperscript{-1}. The position of these bands suggests that complex is due to \[\text{B}_{\text{II}}\] \[\rightarrow\] \[\text{E}_{\text{II}}\] and \[\text{B}_{\text{II}}\] \[\rightarrow\] \[\text{A}_{\text{II}}\] transitions [18]. Various ligand field parameters were calculated as transitions and a square planar stereochemistry. The above arguments indicate that the ligand in complex behaves as a neutral bidentate ligand and coordination occurs via the carbonyl oxygen and azomethine nitrogen atoms.

Free radicals study

Data presented in (Table 1) showed nitric oxide and malonaldehyde levels among different studied groups. Normal mice treated with the copper complex showed a significant increase in liver nitric oxide from (10.59±0.29) to (10.88±0.41), as well as liver MDA from (6.44±0.21) to (6.53±0.25) and erythrocyte MDA from (4.25±0.27) to (5.53±0.32), (p < 0.05) compared to normal mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Liver MDA</th>
<th>Erythrocyte MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/mg protein</td>
<td>µmol/mg protein</td>
<td>µmol/µg Hb</td>
</tr>
<tr>
<td>1</td>
<td>10.59±0.29</td>
<td>6.44±0.21</td>
<td>4.25±0.27</td>
</tr>
<tr>
<td>2</td>
<td>9.9±0.65</td>
<td>6.7±0.35</td>
<td>4.75±0.30</td>
</tr>
<tr>
<td>3</td>
<td>10.88±0.41*</td>
<td>6.53±0.25*</td>
<td>5.53±0.32*</td>
</tr>
<tr>
<td>4</td>
<td>12.59±0.29</td>
<td>11.31±0.85</td>
<td>10.69±0.33</td>
</tr>
<tr>
<td>5</td>
<td>10.92±0.41*</td>
<td>8.44±0.32*</td>
<td>8.44±0.32*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD of ten mice (N=10).

\(*\) Significant (p < 0.05) compared to normal control group.

\(\$\) Significant (p < 0.05) compared to tumor-bearing mice group.

Antioxidant enzymes study

Data in Table 2 showed that treatment of normal mice with the copper complex had no significant change in the liver SOD, GPX, activities and reduced GSH. In tumor-bearing mice liver SOD and GPX activities as well as reduced GSH level was lower than that found for normal mice by 31.5 %, 42.4 % and 38.6 % (p < 0.05) respectively. In tumor-bearing mice treated with copper complex, SOD and GPX activities were significantly higher than those of tumor-bearing mice by 13.4 %, 25.5 % (p < 0.05) respectively. As well as, the reduced glutathione was significantly higher than those of tumor-bearing mice treated with copper complex by 22.4 % (p < 0.05), compared to tumor-bearing mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD U/mg protein</th>
<th>GSH µmol/mg protein</th>
<th>GPX U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>692.9±0.426</td>
<td>17.31±0.81</td>
<td>79.1±2.01</td>
</tr>
<tr>
<td>2</td>
<td>679.0±0.76</td>
<td>15.0±0.23</td>
<td>73.8±1.92</td>
</tr>
<tr>
<td>3</td>
<td>680.5±0.80</td>
<td>16.0±0.27</td>
<td>71.3±0.95</td>
</tr>
<tr>
<td>4</td>
<td>474.3±1.47</td>
<td>10.62±0.23</td>
<td>43.8±0.52</td>
</tr>
<tr>
<td>5</td>
<td>53.8±0.76</td>
<td>13.0±0.37</td>
<td>35.0±8.09</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD of ten mice (N=10).

\(*\) Significant (p < 0.05) compared to normal control group.

\(\$\) Significant (p < 0.05) compared to tumor-bearing mice group.

Histopathological study of liver

The histomorphological changes seen in the cytoplasm and nuclei of the liver cells of tumor-bearing mice treated with saline or copper complex are shown in Figs. 1-4. The histopathological examination of liver section in control mice group injected with saline solution showed normal pattern of central vein, hepatic lobules, hepatic cells, kupffer cells, binucleated cells and blood sinusoids (Fig. 1) compared to control mice groups injected with complex solvent which showed dilated congested central vein, enlarged swollen hepatic lobule, vacuolar degeneration of hepatocytes and lymphocytic infiltration (Fig. 2).

While liver section in control mice group induced with Earlich ascites carcinoma cells showed dilated central vein, enlarged swollen hepatocytes and hepatic lobules, dilated blood vessels vacuolar degeneration of hepatocytes and lymphocytic infiltration (Fig. 3). Liver of mice group induced with Earlich ascites carcinoma cells and treated with copper complex showed dilated blood diluted blood sinusoids which reveal an improvement.
DNA content and cell cycle study

Data represented (Table 3) showed that G0-G1 in copper complex EAC treated group exhibited a significant increase compared to EAC control group. Concerning to total S-phase, copper complex EAC cells treated groups exhibited a significant decreased compared to EAC control group.

Table 3. DNA content and cell cycle study of EAC of both control and treated groups.

<table>
<thead>
<tr>
<th>Phase Cell Cycle</th>
<th>EAC Control Group</th>
<th>EAC Treated Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid G0-G1</td>
<td>35.27% at 37.68</td>
<td>45.22% at 46.38</td>
</tr>
<tr>
<td>Diploid G2-M</td>
<td>20.47% at 75.36</td>
<td>17.95% at 92.76</td>
</tr>
<tr>
<td>Diploid S</td>
<td>44.26%</td>
<td>36.82%</td>
</tr>
<tr>
<td>Diploid G2/G1</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Diploid % CV</td>
<td>11.44</td>
<td>15.06</td>
</tr>
<tr>
<td>Total S-phase</td>
<td>44.26%</td>
<td>36.82%</td>
</tr>
<tr>
<td>S-phase Assessment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid S</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Calculated P-Value</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>S-phase Boundaries</td>
<td>5.0 and 10.0</td>
<td>5.0 and 10.0</td>
</tr>
<tr>
<td>Modeled Events</td>
<td>13948</td>
<td>15366</td>
</tr>
</tbody>
</table>

DISCUSSION

It is well known that cancer cells biochemically differ from normal cells in many manners. Cancer cells have altered patterns of SOD activity from those seen in normal cells. It has been found that cancer cells usually, but not always, have lowered levels of CuZn-SOD activity and always have lowered levels of Mn-SOD activity when compared to the level in an appropriate control [19]. Thus, a large of different compounds with SOD-like activities has to be tested as antitumor drugs. In the present study SOD model compounds such as [CuL(OH)][(ClO₄)] complexes were investigated. These complexes were found to scavenge O²⁻ in the order: [CuL(OH)][(ClO₄)].

The concurrent study revealed that nitric oxide and malondialdehyde levels in both liver homogenate and erythrocyte lysate exhibited a significant decrease in EAC group treated with copper and cobalt complexes compared to EAC group without treatment. EAC group without treatment showed a significant increase compared normal control group. These results are in agreement with Kern and Kehrer [20]. They revealed that cumulative production of ROS typical for many cancer cells is linked with altered redox regulation of signaling cascades. The reducing intracellular environment in the nucleus and in mitochondria (maintained by elevated levels of glutathione and thioredoxin) not only facilitates escape from apoptosis but also produces proliferation potential through activation of cell survival signals mediated by redox-sensitive nuclear transcription factors. Reactive oxygen species have multiple functions [21] and are implicated in tumor initiation and progression as well as in induction of apoptosis of various cancer cells [22]. Often, the ability of a therapeutic agent to induce apoptosis of cancer cells depends upon the ability of those cancer cells to generate ROS [23].

Although reactive oxygen species is important for cell signaling and different physiological processes [24], too much ROS level and oxidative stress is harmful [25]. Interestingly, CuNG was found to have no suppressive effect on lympho proliferation and was rather found to reduce tumor-induced immunosuppression [26]. Copper accumulation may lead to toxicity [27]. Since CuNG is a copper complex, it increases the level of Cu in the system. It has been reported that serum copper level was elevated in animals and humans with cancer [28]. The
increase of liver Cu was found to be inversely proportional to liver-GSH level; the level of GSH has been found to be depleted by 50% after the injection of CuNG. Depletion of GSH by Cu has previously been reported e.g., by CuSO_4 [29]. Cu is stored mostly as metallothionein (MT)-copper complexes in the organism while the amounts of unbound free Cu is almost negligible; upon copper injection, GSH binds to Cu before the metal complexes with MT [30]. The initial depletion of GSH with increase in Cu level in the liver induced by CuNG may be due to the formation of GS-CuNG complexes [31].

In the present study SOD, GSH and GPX levels in both liver homogenate exhibited a significant decreased in EAC group treated with copper complex compared to EAC group without treatment, at the same exhibited a significant increase compared to normal control. Superoxide is a major factor in tumor promotion, and re-perfusion injury [32]. Wenqing [33] showed an effective defense system against the toxicity of O_2^-, such as superoxide dismutase and catalase. Generally there are three groups of SOD; each has different metal in the active site. According to the metals, they are termed as CuZnSOD, MnSOD and FeSOD. Acting as one of the most important antioxidants, all SODs catalyze the dismutation of O_2^-

CuZnSOD contains two protein subunits; each of its active site contains one copper and one zinc ion. The zinc ion acts to stabilize the enzyme, while the copper ion acts as the functional metal. Since copper acts as the active center in CuZnSOD, many copper complexes has been synthesized and tested. Research has shown that many complexes have SOD- like activities, such as Cu(II)(3,5-diisopropylsalicylic acid)_2 (CuDIPS) [36], Cu(II) histidine complexes [37], Cu(II) complexes of macrocyclic polyamine derivatives [38], Bis(2,9-dimethyl-1,10-phenanthroline)-Cu(II)nitrate (Cu(II)(DMP)) [38] and Cu(II)-oligopeptide. Since the dismutation reaction involves the redox cycle of Cu (II) and Cu (I), it is reasonable to expect that the redox potential of Cu (II) complex/Cu (I) complex can influence the SOD-like activity, while the ligand of the complex determines the redox potential [39]. It was shown that although His-Phe-Cu (II) complexes had a relatively high SOD activity. Kimura et al. proposed that the activity depended on ring size, type and subsistent on the macrocycles. Although in vitro studies show promising results of these copper complexes, in vivo experiments are often disappointing. The first reason is that many complexes undergo dissociation in vivo, yielding copper ions that subsequently combine with serum components and lose their SOD-like activity. The second reason relates to the reoxidation of the reduced compound by O_2.

Copper has been reported to induce apoptosis by generation of ROS, and since CuNG is a copper (II) chelate, it warranted a study on its effect on ROS generation, which includes beside GSH, a number of antioxidant enzymes, like GPx, SOD and CAT [39]. It has been shown that the cell cycle is characterized by fluctuations in the redox environment of a cell, mediated, in particular by intracellular changes in concentration of glutathione. Oxidizing molecules, such as H_2O_2 and thioredoxin are present outside the cell; H_2O_2 can cross the plasma membrane and enter the cells, leading to changes in the intracellular redox environment. In addition, membrane proteins, such as NADPH oxidase on neutrophil membranes, can produce H_2O_2. Induction or inhibition of cell proliferation seems to be dependent on levels of oxidants/antioxidants in the cell. A more reducing environment of the cell stimulates proliferation and a slight shift towards a mildly oxidizing environment initiates cell differentiation. A further shift towards a more highly oxidizing environment in the cell leads to apoptosis and necrosis. Apoptosis is induced by moderate oxidizing stimuli and necrosis by an intense oxidizing effect.

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