Stability, Toxicity and Cytotoxicity of a Cupric Complex Towards Cultured CaCo-2 Cells

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Abstract. The human colon adenocarcinoma-derived cell line CaCo-2 was used as a model system to study the effects of copper (II), tetradentate N-alkyl ligand (H2L2) and their complex. The stimulatory effect of the complex was obtained with lower concentrations from 10⁻⁷ to 10⁻⁵ mol L⁻¹ while inhibiting effects occur from 10⁻⁴ to 10⁻³ mol L⁻¹. According to this study, copper (II) and free ligand were toxic for cultured cells. Our data suggested that the complex, according to our toxicity assays in mice, could be used as an antiinflammatory agent.

We recently described the potent cytotoxicity of the complex [Cu(H2L)(H2O)2]Cl2, 4H2O (H2L-N,N'-bis(2-hydroxyacetophenyl)-1,2-diaminoethane) towards various leukemias, lymphomas and carcinoma cells [1]. We have demonstrated that this complex causes a 10 % reduction in DNA topoisomerase II inhibition at 150µM. In an attempt to obtain metallic complexes with the aim of enhancing copper (II) effects against cancer with less toxicity, we have already studied in mice, the toxic effects of metallic complexes obtained with cupric ions and 3-(2-furyl) prop-2 enal semicarbazone [2]. In our continuous investigations concerning new antimitic metal complexes, we have described the stability versus time of the copper salt (CuCl2), the free linear tetradentate ligand and their complex [3]. We also determined and compared their cytotoxicity in cultured CaCo-2 cells and their toxicity in mouse Swiss mice.

Materials and Methods

Chemicals. The synthesis and the chemical characterization of the ligand (Figure 1) N,N'-bis(2-hydroxyacetophenyl)-1,2-diaminoethane (H2L)

and the complex [Cu(H2L)(H2O)2]Cl2, 4H2O have been previously described [3]. Copper (II) chloride CuCl2, 2H2O of analytical grade was purchased from Fluka and used without further treatment.

The HPLC chromatograms were recorded using Rosorb OD315 C8 column with Gilson and Soparops pumps and Gilson autosampling injector; the detector was UV-Spectromonitor D (LDC/Milton Roy) at 210 nm. Dulbeco's modified Eagle essential medium (DMEM) was from Jacques Boy, France. Other biological chemicals were from Eurobio, France.

Cells cultures. CaCo-2 cells, from passage 75 to 100 were routinely cultured in a culture flask at 37 °C in a humid atmosphere obtained with 90 % of O₂ and 10 % of CO₂ in DMEM containing 20 % fetal calf serum, 1 % penicillin-streptomycin and 1 % non essential aminoacids (AANE). The cultures were fed every 48 hours. The medium was changed and new treatments were added to the cell cultures every 48 hours. The tested molecules were added to the cultures in aqueous solutions. The cells were trypsinized and counted using an hemocytometer.

Cytotoxic activities. To perform cytotoxic activity, cells were plated in 96-well tissue culture plates (10⁵ cells/cm²). After overnight growth, the medium was changed to DMEM with studied molecules at various concentrations. Cells were washed with phosphate buffer saline (PBS), fixed with 20 % (V/V) trichloroacetic acid and the plates were washed with PBS five times and then dried for 30 minutes. Sulfurobacin B (SRB) in acetic (0.04 % w/w) was added over 30 minutes. The plates were rinsed three times with 1 % acetic acid. SRB was extracted with tris-base buffer at pH = 10.4. The incorporation of SRB to the cells depended on protein synthesis.

Toxicity test. Male Swiss mice from Debré (France) weighing from 20 to 25 g were used because of their regular weight gain and the absence of oestral cycle with changes in sexual hormonal status, which could lead to some errors in vivo experiments. The animals had free access to both water and a standard diet containing 0.8 % Ca, 0.7 % P and 3 ULg⁻¹ vitamin D. They were placed at room temperature (20 ± 2 °C) and exposed to a 12 hour light / 12 hour dark cycle. After being injected with various doses of studied complexes, the animals were kept under the same conditions and were followed for 7 days.

Results

Complex stability. The HPLC chromatograms of all complexes, are presented in Figure 2 and Table I summarizes

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the results of the study of the stability of the complex when stored respectively at 4°C and 37°C. As the retention time and the peak area remained quite unchanged at 4°C as well as at 37°C, the complex was stable for at least a week and could be used for biological tests.

**Cytotoxic tests.** The main results are presented in Table II. It may be noted here that mitosis of cultured CuCo-2 cells was inhibited by concentrations higher than $10^{-8}$ mol.L$^{-1}$ (CuCl$_2$), $10^{-6}$ mol.L$^{-1}$ (ligand) and $10^{-6}$ mol.L$^{-1}$ (complex).

**Toxicity assays.** 1 - copper chloride: The effects of CuCl$_2$ on male Swiss mice showed that this salt is a very toxic compound. The lethal dose 0 (LD 0) was 5µg.kg$^{-1}$ body weight (BW); LD 25 was 15 µg.kg$^{-1}$ BW; LD 50: 25 µg.kg$^{-1}$ BW; LD 75: 35 µg.kg$^{-1}$ BW and finally LD 100 was 45µg.kg$^{-1}$ BW.

2 - ligand: A dose up to 45µg.kg$^{-1}$ BW, did not kill any mice 7 days after the injection.

3 - complex: The results obtained were the same as with the ligand.

**Discussion**

According to our results, the complex [Cu(H$_2$L)(H$_2$O)$_2$]Cl$_2$. 

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**Table 1. Retention times (RT) and peak areas (PA) for the stability study of the complex.**

<table>
<thead>
<tr>
<th>Time</th>
<th>4°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT (min)</td>
<td>PA</td>
</tr>
<tr>
<td>9 h</td>
<td>8.44</td>
<td>1005</td>
</tr>
<tr>
<td>11 h</td>
<td>8.33</td>
<td>952</td>
</tr>
<tr>
<td>13 h</td>
<td>8.15</td>
<td>1003</td>
</tr>
<tr>
<td>15 h</td>
<td>8.75</td>
<td>1017</td>
</tr>
<tr>
<td>17 h</td>
<td>9.05</td>
<td>1027</td>
</tr>
<tr>
<td>24 h</td>
<td>8.71</td>
<td>1042</td>
</tr>
<tr>
<td>2 days</td>
<td>8.54</td>
<td>1015</td>
</tr>
<tr>
<td>3 days</td>
<td>8.13</td>
<td>965</td>
</tr>
<tr>
<td>4 days</td>
<td>7.94</td>
<td>899</td>
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<tr>
<td>5 days</td>
<td>8.47</td>
<td>955</td>
</tr>
<tr>
<td>6 days</td>
<td>8.74</td>
<td>1069</td>
</tr>
<tr>
<td>7 days</td>
<td>8.00</td>
<td>875</td>
</tr>
</tbody>
</table>
4H2O was stable at 4 °C and 37 °C for 7 days. Retention times and peak areas remained quite unchanged (Table I and Figure 2). This is a very important result as the CaCo-2 cells were cultured at 37 °C for at least 48 hours with copper (II) chloride, ligand and complex dissolved in water and then stored at 4 °C.

We already tested cobalt (II) cation as anticancer treatment and some interesting results were obtained [4, 5]. The copper (II) cation is the main cofactor for many enzymatic systems and has properties against oncogenesis by protecting from free radical effects [6]. Unfortunately, cupric chloride is toxic by itself and cannot be used as an anticancerogenisis. CaCo-2 cells are a cell line derived from human colon adenocarcinoma of malignant origin [7] and the complex [Cu(H2L)(H2O)2]Cl2. 4H2O was used to control colorectal cancer cell growth.

Figure 3 shows that the ligand and copper (II) inhibited cell proliferation from 10−4 to 10−17 mol.L−1, while the complex stimulated cancer cultured cell mitosis in the range 10−2−10−17 mol.L−1 and inhibited cell proliferation from 10−6 to 10−6 mol.L−1. These facts suggest that the complex is less toxic than the free ligand and CuCl2 in cultured cells. The observations reported in the present study corroborate our previous work in mice [5]. Copper (II) has a LD100 of up to 44 µg.kg−1 (body weight) while the ligand and its complex do not kill any animal at this dose. Finally, we can assume that the complex

<table>
<thead>
<tr>
<th>Concentration (mol.L−1)</th>
<th>Copper (II) chloride</th>
<th>Ligand</th>
<th>Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>10−4</td>
<td>73 ± 5</td>
<td>71 ± 6</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>10−5</td>
<td>85 ± 7</td>
<td>97 ± 8</td>
<td>70 ± 14</td>
</tr>
<tr>
<td>10−6</td>
<td>85 ± 3</td>
<td>105 ± 4</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>10−7</td>
<td>90 ± 10</td>
<td>104 ± 9</td>
<td>98 ± 11</td>
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<tr>
<td>10−8</td>
<td>104 ± 16</td>
<td>104 ± 4</td>
<td>101 ± 14</td>
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<tr>
<td>10−9</td>
<td>102 ± 10</td>
<td>106 ± 4</td>
<td>102 ± 16</td>
</tr>
<tr>
<td>10−10</td>
<td>106 ± 7</td>
<td>111 ± 40</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>10−11</td>
<td>143 ± 22</td>
<td>157 ± 40</td>
<td>112 ± 28</td>
</tr>
<tr>
<td>10−12</td>
<td>161 ± 17</td>
<td>186 ± 25</td>
<td>138 ± 19</td>
</tr>
<tr>
<td>10−13</td>
<td>155 ± 31</td>
<td>135 ± 11</td>
<td>133 ± 17</td>
</tr>
<tr>
<td>10−14</td>
<td>161 ± 16</td>
<td>128 ± 8</td>
<td>136 ± 9</td>
</tr>
<tr>
<td>10−15</td>
<td>140 ± 20</td>
<td>168 ± 24</td>
<td>135 ± 11</td>
</tr>
<tr>
<td>10−16</td>
<td>121 ± 9</td>
<td>160 ± 21</td>
<td>144 ± 26</td>
</tr>
<tr>
<td>10−17</td>
<td>144 ± 8</td>
<td>141 ± 28</td>
<td>147 ± 14</td>
</tr>
</tbody>
</table>
[Cu(H2L)(H2O)2]Cl2. 4H2O (H2L = N,N'-bis(2-hydroxyacetophenyl)-1,2-diaminoethane) can be used to inhibit CaCo-2 cell growth because of its lower toxicity.

For further experiments, we must take into account these data and deal with mice presenting induced colorectal cancer. Studies must be done with intact mice as control and the effects of copper (II) chloride, free ligand and complex must be followed for some months after intraperitoneal injection and/or oral administration.

References


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