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Dna Cleavage and Cytotoxic Activity of Copper(II) Complexes Based on Reduced Schiff Bases Derived From Salicylaldehyde and Amino Acids

Short communication

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Abstract Metal complexes, which, under physiologic conditions, show redox properties and are able to bind to DNA, are great tools to cleave the DNA chain. This aspect is of great importance for their use as antineoplastic drugs. We synthesized ligands derived from short-chain amino acids and from salicylaldehyde. The prepared ligands of the type of reduced Schiff bases were subsequently used for the preparation of copper(II) complexes. The aim of the study was in vitro testing of copper(II) complexes, where it was confirmed that they are capable of cleaving DNA. Their cytotoxic activity was also confirmed by the resazurin redox method on Saccharomyces cerevisiae based on preserved healthy mitochondrial function.

Keywords *copper(II) complexes – anticancer drugs – DNA cleavage activity – cytotoxicity*

INTRODUCTION

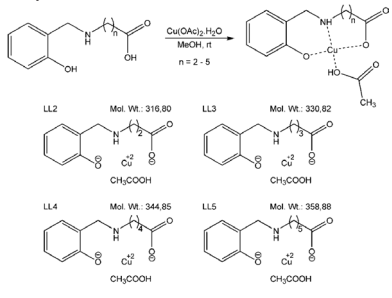
In recent years, a large number of synthetic copper(II) complexes have been reported to act as pharmacologic agents and as potential anticancer and cancer-inhibiting agents, and they have been found to be active both *in vitro* and *in vivo* (1). Their biological activity could be related to the binding to DNA via both covalent and noncovalent interactions and the redox properties of copper. Copper has two energetically similar oxidation states (+I/+II) that can exist under physiologic conditions and in the presence of oxygen species, it can accept or give an electron depending on the oxidation state that copper is in. This enables antioxidant or prooxidant behavior of copper and significantly propagates reactive oxygen species (ROS), which could subsequently induce DNA breakages. Thus, some cytotoxic copper compounds often show DNA cleavage activity (2). Not long ago have been reported any Cu(II) compounds derived from Schiff base thiosemicarbazones exerting significant cytotoxicity against cancer cell lines *in vitro* and inducing cancer cell apoptosis through the intrinsic ROS-mediated mitochondrial pathway (3). Among the many bio-essential metals, copper complexes are regarded as promising alternatives to platinum complexes as anticancer drugs because copper is biocompatible and exhibits many significant roles in biological systems (4).

MATERIALS AND METHODS

Synthesis of copper(II) complexes

Synthesis of ligands was performed in two steps. In the first step, Schiff bases were prepared from salicylaldehyde and sodium salts of linear amino acids (β-alanine, γ-aminobutanoic acid, 5-aminopentanoic acid, and 6-aminohexanoic acid) by condensation reaction. In the second step, a reduction of the imine double bond with $NaBH_4$ was performed to obtain reduced Schiff base ligands. The copper(II) complexes were prepared from reduced Schiff bases and copper(II) acetate in methanol as green powders. The prepared ligands were characterized by nuclear magnetic resonance (NMR), IR, and elemental analysis, and the complexes were characterized by IR and elemental analysis.

Scheme of synthesis of copper(II) complexes and the listed prepared complexes:



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3-(2-Hydroxy-benzylamino)-β-alanine (free ligand from LL2). Elemental analysis calculated for $C_{10}H_{13}NO_3$ (molecular weight [MW] = 195.22) C 61.53; H 6.71; N 7.18, measured C 61.43; H 6.52; N 7.09. ^{1}H NMR (300 MHz, DMSO-d_6, ppm) δ: 7.05–7.10 (m, 2H), 6.77 (dd, 1H), 6.69 (m, 1H), 3.86 (s, 2H), 2.72 (t, 2H), 2.20 (t, 2H). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ: 175.4, 157.8, 129.3, 128.4, 122.6, 118.0, 115.6, 49.1, 44.9, 35.0. IR (solid, cm^{-1}): 3408, 3163, 2988, 2789, 1716, 1610, 1599, 1506, 1461, 1406, 1380, 1247, 1187, 1113, 1081, 1025, 982, 946, 896, 759, 660, 649, 608

4-(2-Hydroxy-benzylamino)-butanoic acid (free ligand from LL3). Elemental analysis calculated for $C_{11}H_{15}NO_3$ (MW = 209.24) C 63.14; H 7.23; N 6.69, measured C 63.34; H 7.45; N 6.62. ^{1}H NMR (300 MHz, DMSO-d_6, ppm) δ: 7.06–7.12 (m, 2H), 6.71–6.75 (m, 2H), 3.82 (s, 2H), 2.60 (t, 2H), 2.25 (t, 2H), 1.65–1.69 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ: 178.3, 154.0, 129.8, 129.4, 122.2, 120.6, 115.9, 47.9, 42.7, 30.0, 20.0. IR (solid, cm^{-1}): 3067, 2951, 2886, 2743, 2631, 1645, 1594, 1502, 1472, 1456, 1434, 1392, 1357, 1333, 1303, 1276, 1250, 1180, 1155, 1105, 1039, 1020, 944, 915, 862, 845, 762, 731, 667, 638, 604

3-(2-Hydroxy-benzylamino)-β-alaninato copper(II) acetic acid complex (**LL2).** Elemental analysis calculated for $C_{12}H_{15}CuNO_5$ (MW = 316.80) C 45.50; H 4.77; N 4.42, measured C 45.14; H 4.90; N 4.34. IR (solid, cm^{-1}): 3139, 2911, 2710, 2604, 1611, 1596, 1504, 1463, 1432, 1398, 1353, 1265, 1239, 1186, 1117, 1047, 942, 871, 860, 761, 747, 683, 673, 653, 642, 630, 609

4-(2-Hydroxy-benzylamino)-butanoato copper(II) acetic complex (**LL3)**. Elemental analysis calculated for $C_{13}H_{17}CuNO_5$ (MW = 330.82) C 47.20; H 5.18; N 4.23, measured C 46.96; H 5.05; N 4.11. IR (solid, cm^{-1}): 3180, 2940, 1599, 1569, 1483, 1455, 1436, 1424, 1399, 1308, 1290, 1266, 1238, 1111, 1066, 1038, 977, 931, 918, 900, 877, 841, 781, 752, 728, 685, 640

The complexes (**LL4)**, (**LL5**), and their free ligands were prepared by Dr. Lucia Lintnerová and have been published in a previous study. (5)

DNA cleavage activity

Two hundred and fifty nanograms of DNA plasmid pBBR322 (in 25 mM Tris HCl buffer solution, pH 7.5) was treated with copper(II) complexes (suspended in DMSO) for 5 h at 37 °C. Each reaction was then quenched by adding 4 µL of a Smart Glow Loading Dye® (0.01% Safe Green dye, 50% glycerol, and 250 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0), and the digested samples were electrophoresed on 0.9% agarose gel containing TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at 55 V/40 mA for about 1.5 h. TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) was used as the "running buffer." The resulting cleaved fragments which correspond to the cleaved form II. (open circular form) and cleaved form III. (linear form) were visualized by ultraviolet (UV) light (6,7). Quantitative evaluation of cleavage activity was performed using the "gelQUANT software" (Cleaver Scientific, UK).

Cytotoxic activity of copper(II) complexes

Saccharomyces cerevisiae (3,8) cells (CCY 21-4-64; Libáň, Czech Republic) were cultured on Sabouraud dextrose agar with chloramphenicol (Merck, USA) and liquid YPD medium (Sigma-Aldrich, USA) at 28°C/24 h. In the exponential growth phase, the cells were centrifuged (8,000 *g*/3 min), washed twice with physiologic solution (FS 0.9% NaCl), and pipetted at a concentration of 1×10^9 cells/ml into sterile microtubes. Sterile FS and copper(II) complexes (1×10^{-3} M, 3×10^{-3} M, and 5×10^{-3} M) were added to the centrifuged biomass and cultured at 28°C/3 h. After 3 h, 0.1 ml of resazurin (Sigma, USA) was added to the samples and they were incubated for 2 h. After 2 h, the biomass was separated from the supernatant by centrifugation (8,000 *g*/5 min). The separated solutions were diluted in a ratio of 1:1 with FS and measured (600 nm/resazurin, 570 nm/resorufin = metabolized resazurin) on UV-VIS spectrophotometer (Thermo Scientific, USA) using a 1-cm-long cuvette (8,9). Control samples used: C– negative control (1 ml 0.9% NaCl + 0.1 ml resazurin); C+ positive control (*S. cerevisiae* cells 1×10^9/ml + 1 ml 0.9% NaCl + 0.1 ml resazurin) measured at t = 3 h.

Experimental parameters

Cells' innate metabolism was measured by using an established formula for reduction percentage. Reduction percentage was calculated by the following formula:

$$\text{Red [\%]} = \frac{\mathcal{E}_{OX\_600\ nm} \times A_{570\ nm\_sample} - \mathcal{E}_{OX\_570\ nm} \times A_{600\ nm\_sample}}{\mathcal{E}_{RED\_570\ nm} \times A_{600\ nm\_C-} - \mathcal{E}_{RED\_600\ nm} \times A_{570\ nm\_C-}} \times 100$$

The cytotoxic activity of copper(II) complexes was calculated according to the formula

$$\text{Cy [\%]} = 100 - \text{Red [\%]}$$

Molar extinction coefficients for reduced and oxidized resazurin:

Wavelenght	Resorufin $\mathcal{E}_{RED}$	Resazurin $\mathcal{E}_{OX}$
570 nm	155,677	80,586
600 nm	14,652	117,216

RESULTS AND DISCUSSION

DNA cleavage activity

Nuclease activity was recorded for all tested complexes, which increased predominantly with increasing concentration of the copper complex. In complexes LL2, LL3, LL4, and LL5,

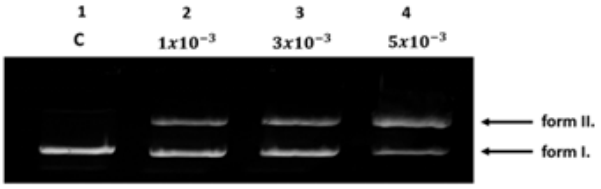

Fig. 1. DNA cleavage activity of LL2 complex after 5h/37°C incubation
Line 1: C- negative control (pBR 322 in TRIS-HCl, pH 7.5)
Line 2-4: LL2 in the concentration of $1x10^{-3}, 3x10^{-3}, 5x10^{-3}$ added to pBR 322.

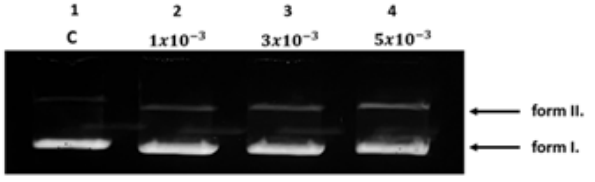

Fig. 2. DNA cleavage activity of LL3 complex after 5h/37°C incubation
Line 1: C- negative control (pBR 322 in TRIS-HCl, pH 7.5)
Line 2-4: LL3 in the concentration of $1x10^{-3}, 3x10^{-3}, 5x10^{-3}$ added to pBR 322.

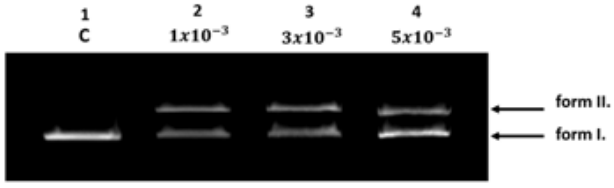

Fig. 3. DNA cleavage activity of LL4 complex after 5h/37°C incubation
Line 1: C- negative control (pBR 322 in TRIS-HCl, pH 7.5)
Line 2-4: LL4 in the concentration of $1x10^{-3}, 3x10^{-3}, 5x10^{-3}$ added to pBR 322.

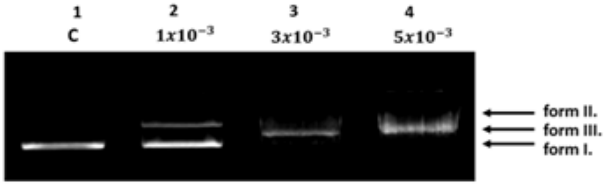

Fig. 4. DNA cleavage activity of LL5 complex after 5h/37°C incubation
Line 1: C- negative control (pBR 322 in TRIS-HCl, pH 7.5)
Line 2-4: LL5 in the concentration of $1x10^{-3}, 3x10^{-3}, 5x10^{-3}$ added to pBR 322.

plasmid DNA (pDNA) was split into an open circular form (form II.) in the amount of LL2: 35.7%–73.8% (1×10^{-3}–5×10^{-3} M); LL3: 7.8%–15.2% (1×10^{-3}–5×10^{-3} M); LL4: 37.1%–47.9% (1×10^{-3}–5×10^{-3} M); LL5 21.3% (1×10^{-3} M). Complex LL5 had the best cleavage activity (compared to negative control), which, in the concentration of 3×10^{-3} and 5×10^{-3} M, completely cleaved pDNA into a linear form (100% form III.) from the original double-stranded supercoiled form (form I.) of pDNA (Figs 1–4).

Cytotoxic activity of copper(II) complexes

The cytotoxic activity of the individual complexes is shown in Fig. 5. All tested copper complexes showed a high cytotoxic activity above the level of 90% compared to the positive control (8%). Cytotoxicity test is based on the ability of living cells (redox reaction in mitochondria) to reduce blue resazurin (7-hydroxy-3*H*-phenoxazin-3-one 10-oxide) into pink resorufin (7-hydroxy-3*H*-phenoxazin-3-one) (8,10). Bioreduction of the dye by viable cells reduces the amount of the oxidized form (blue) and concomitantly increases the amount of its fluorescent intermediate (pink), indicating the degree of cytotoxicity caused by the test material (2,11). Cytotoxic activity was recorded for all tested copper(II) complexes in concentrations of 1×10^{-3}, 3×10^{-3}, and 5×10^{-3} after only 3 h of incubation with *S. cerevisiae* cells.

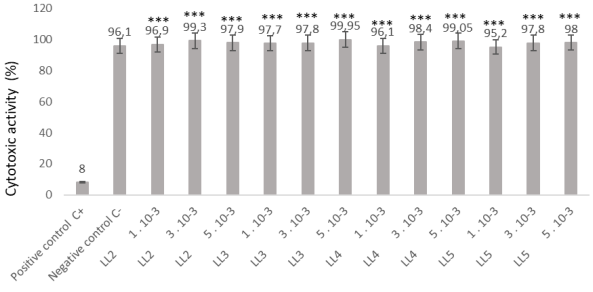

Figure 5. Cytotoxic activity of copper(II) complexes. Expressed as a percentage of the amount of untransformed resazurin to resorufin after 3 h of incubation with copper(II) complexes. Comparison with C+ control was performed by t-test Significance levels: $^{***}0.000 < P < 0.001$; $^{**}0.001 < P < 0.01$; $^{*}0.01 < P < 0.05$

CONCLUSION

In our work, all tested copper complexes showed a high cytotoxic activity above the level of 90% compared to the positive control (8%); also, DNA cleavage activity was recorded for all tested complexes. We assume that the stronger cytotoxic activity of the copper(II) complexes compared to their DNA cleavage activity (especially, in the case of the LL5) is caused by a different mechanism of action on the cell. The complexes significantly affected the metabolic center of the cell, while their effect on DNA may be related to their different structure and ability in some cases to form larger dimers. These structures are larger and their ability to integrate into DNA is greatly hindered. The mechanism of action of copper(II) complexes is still under research. Metal complexes, which, under physiologic conditions, have the ability to bind and cleave stranded DNA, are of great importance for their utility as antineoplastic drugs. A series of copper(II) complexes, exhibiting higher DNA-binding affinity, efficient DNA cleavage, and potent cytotoxicity, which arrested the cell cycle progression leading to apoptotic mode of cell death, were investigated.

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