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Synthesis, physico-chemical properties and biological analysis of newly obtained copper(II) complexes with pyrazole derivatives

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ABSTRACT

Three new copper(II) complexes containing two different pyrazole bound ligands (**1**, **2**) have been synthesized and characterized by IR, LSI-MS (liquid secondary ion mass spectrometry) and elemental analysis. ^{1}H NMR spectra of the organic ligands have been recorded. We describe the influence of these complexes on particular cancer cell lines and DNA structure by MTT-assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], APA (acid phosphatase activity)-assay or CD-spectroscopy and agarose gel electrophoresis methods, together with their physico-chemical properties such as lipophilicity and stability in aqueous solution. The cytotoxic effect on HUVEC (endothelial cells) for the most active complex **4** has been also investigated. Moreover, the ability of these complexes to induce apoptosis in cancer cells has been assessed by using fluorescence microscopy. Our results indicate that dichloridobis{1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1*H*-pyrazole-κN2}copper(II) is the most potent complex among the tested complexes.

1. Introduction

Almost every second person diagnosed with cancer dies due to non-effective therapy [1]. Chemotherapy, surgery and radiotherapy are general directions in cancer treatment. For many years, laboratories all over the world have been conducting research on new and more effective drugs that show fewer side effects. Despite substantial progress in the field, no compound has been developed that satisfies the expectations of both, patients and doctors. This problem relates to the resistance of many types of cancer to drugs, to undesirable side effects caused by currently prescribed drugs, to their low specificity, or to the necessity of using many different cytostatic drugs in long-term combination treatment [2]. Therefore, the search of new compounds with anticancer activity is one of the fundamental tasks of medicinal chemistry [3].

Many metal complexes that are produced by our organism or ingested through food are essential for our well being. Some of them act as anticonvulsants, others as neuronal messengers and again others are involved in the catalysis of enzymatic pathways of some metabolic processes [4].

Transition metal complexes are an interesting group of compounds, from which several anticancer drugs are derived. The variety of their chemical structure and properties gives a wide spectrum of possibilities in synthesis of compounds with different specificity or mode of action. It is stated that the addition of metal ion to an organic ligand with biological activity can lead to an increase of its properties [5,6]. Moreover, pharmacologic properties of complexes can depend on the properties of either metal or ligand or both [7–10].

It is known that many metal complexes with different organic ligands possess a distinct cytotoxicity against various cancer cell lines [6,11]. Copper complexes, as non-platinum drugs, with thiosemicarbazides were tested as a potential antitumor drug in early 1960s and the first related study had been presented by Padhye and Kauffman [12]. In the following years, some copper(II) complexes with thiosemicarbazone derivatives demonstrated anticancer activity in vitro against human leukemia U973 cells [13] and the ability to induce apoptosis is their main mechanism of action [14]. More recently, many Cu(II)–thiosemicarbazide complexes have been investigated as potential antibacterial or antifungal compounds [15].

In 2001, Easmon et al. [16] showed that modified thiosemicarbazide derivatives possess higher cytotoxic activity and lower toxicity when compared to normal cells. Since then, thiosemicarbazones as well as their complexes have been known for their anticancer properties. They act by inducing apoptosis in cancerous cell lines. Some of these anti-cancer drugs act as apoptosis-inducing agents by activation of

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DNA fragmentation [17]. This work describes the biological activity of pyridoxal thiosemicarbazone [18].

Also, many pyrazole complexes coordinated with metal ions are effective against cancer cells, e.g. pyrazole–rhodium(III) complexes indicate cytostatic activity against HCV29T tumor cells, while their Pd(II) complexes are active against solid-tumor cell lines and in some cases exhibit remarkable activity. It has been suggested that their biological activity depends on the nature of the ligand, the type of the counter ion used and the configuration of the complex [19,20].

Identification of the mode of action of newly obtained compounds is essential for designing an effective new drug. It is stated that the application of compounds with cytotoxic activity to cells can lead to cell cycle stop in the G1 (cell cycle phase G1) phase until all induced damages are fixed or until apoptosis is induced. Necrosis is usually a result of the exposition of cells to compounds in high concentrations [21]. Moreover, these types of compounds can also change the structure of the DNA or destroy the cytoskeleton [22].

For many years our scientific attention has been focused on the investigation of chemical compounds with potential anticancer activity [23,24]. Therefore, in this paper, we present the synthesis as well as the physico-chemical and biological evaluation of newly obtained copper(II) complexes of pyrazole derivatives.

2. Experimental

2.1. General

The IR spectra were recorded with a Mattson Infinity MI-60 spectrophotometer in KBr. Melting points were determined using a Buchi Melting Point B540 apparatus and are uncorrected. Elemental analyses were obtained in the Microanalytical Laboratory of the Department of Bioorganic Chemistry (Medical University, Lodz) using a Perkin Elmer PE 2400 CHNS analyzer. LSI mass spectra (liquid secondary ion) were recorded on the Finnigan MAT 95 double focusing (BE geometry) mass spectrometer (Finnigan MAT, Bremen, Germany). Samples were dissolved in DMSO and 1 μL of 3-nitrobenzylalcohol (NBA) and mixed. For the ionization, the beam of cesium ions of energy of 13 keV was used. Spectra were recorded in positive and negative ion mode. Circular dichroism measurements were performed using a Jasco J-810 spectropolarimeter equipped with a Jasco PFD-4255 Peltier temperature controller. UV-visible (UV–vis) spectra were recorded on a Varian Bio 100 UV–vis spectrophotometer at room temperature.

In all experiments, each compound was dissolved in 10 μL of DMSO and diluted with bidistilled water or Tris–HCl NaCl buffer in order to calculate its concentration. The final percentage of DMSO was 0.1% at most.

2.2. Synthesis of compounds

2.2.1. Synthesis of 1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1H-pyrazole (**1**)

The synthesis of ligand **1** was slightly modified from methods published previously [25,26]. Hydrazinecarbothioamide (2.37 g, 26 mmol) was dissolved in a mixture of ethanol (20 mL) and HCl (1 mL). Next ethyl benzoylacetate was added (4.5 mL, 26 mmol). The obtained mixture was refluxed for 1 h under an argon atmosphere. After cooling, the white precipitate was filtered off, washed with water and dried under reduced pressure. Yield: 3.85 g (67.5%), m.p: 159.1–160.3 °C. IR (KBr) ν (cm^{-1}): 3290, 3159, 3113 (NH_2), 3095 (CH aromat.), 1651 (C═O), 1584, 1558 (C═C, C═N), 1320 (C – NH_2), 1026 (N – N), 1102, 883, and 798 (C═S). ^{1}H NMR (270 MHz, DMSO-d6) δ (ppm): 6.18 (1H, s, C4-H), 7.40–8.04 (5H, m, arom.), 9.50–10.40 (2H, s, NH_2), and 11.75–12.65 (1H, s, OH/NH).

2.2.2. Synthesis of 1-[amino(thioxo)methyl]-3,5-dimethyl-1H-pyrazole (**2**)

Ligand **2** was synthesized according to a known procedure with slight modifications [27,28]. Hydrazinecarbothioamide (2.5 g, 27.4 mmol) was dissolved in HCl (100 mL, 0.05 M) and pentano-2,4-dione (2.5 mL, 24.3 mmol) was added dropwise to the stirred mixture. After stirring for 1.5 h and 3 h incubation at room temperature, a white precipitate was filtered off, washed with water and dried under reduced pressure. Yield: 3.320 g (88.0%), and m.p: 95.2–95.9 °C. IR (KBr) ν (cm^{-1}): 3389, 3241 (NH_2), 3133 (CH_3), 1604, 1574 (C═C, C═N), 1339 (C – NH_2), 1029 (N – N), 1099, 880, and 808 (C═S). ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm): 2.28 (6H, d, – CH_3), 6.29 (1H, s, C4 – H), and 7.00–7.46 (2H, d, NH_2).

2.2.3. Synthesis of dichlorido(1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1H-pyrazole-κN^2)copper(II) (**3**)

A solution of 1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1*H*-pyrazole (**1**) (197.4 mg, 0.9 mmol) in ethyl acetate (5 mL) was added dropwise to a solution of copper(II) chloride dihydrate (153.4 mg, 0.9 mmol) in ethyl acetate (4 mL) and methanol (1 mL) while stirring. The obtained red-orange precipitate was filtered off, washed with diethyl ether and dried under reduced pressure. Yield: 164.0 mg (49.6%), and m.p: 186.1–187.6 °C. IR (KBr) ν (cm^{-1}): 3361 (OH), 3243, 3126 (NH_2), 2987 (CH aromat.), 2363 (SH), 1573, 1520 (C═C, C═N), and 965 (N – N). Anal. Calc. $C_{10}H_9N_3SOCuCl_2 \cdot 3/4H_2O$ (M = 367.229 g/mol) Anal (%): C 32.71, H 2.88, and N 11.44. Found (%): C 32.79, H 2.76, and N 11.25. LSI-MS (*m/z*): 353($LCuCl_2^+$); and 281 (LCu^+).

2.2.4. Synthesis of dichloridobis{1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1H-pyrazole-κN^2}copper(II) (**4**)

While stirring a solution of 1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1*H*-pyrazole (**1**) (131.6 mg, 0.6 mmol) in ethyl acetate (5 mL), a solution of copper(II) chloride dihydrate (51.1 mg, 0.3 mmol) in ethyl acetate (4 mL) and methanol (1 mL) was added dropwise. Next, the mixture was refluxed and stirred for 2 h at 45 °C. The obtained black precipitate was filtered off, washed with ethyl acetate and diethyl ether and dried under reduced pressure. Yield: 87.9 mg (51.2%), and m.p: 160.2–160.9 °C. IR (KBr) ν (cm^{-1}): 3423 (OH), 3152, 3128 (NH_2), 3028 (CH, aromat.), 1606, 1523 (C═C, C═N), 1316 (C – NH_2), 1001 (N – N), 867, and 808 (C═S). Anal. Calc. $C_{20}H_{18}N_6S_2O_2CuCl_2$ (M = 572.984 g/mol) Anal (%): C 41.92, H 3.17, and N 14.67. Found (%): C 41.79, H 3.31, and N 14.27. LSI-MS (*m/z*): 574 ($L_2CuCl_2^+$ + 1H); 538 (L_2CuCl^+); and 219 (L).

2.2.5. Synthesis of dichlorido(1-[amino(thioxo)methyl]-3,5-dimethyl-1H-pyrazole-κN^2)copper(II) (**5**)

Compound **5** was synthesized according to the literature with minor modifications [29]. Copper(II) chloride dehydrate (85.2 mg, 0.5 mmol) in ethyl acetate (4 mL) and methanol (1 mL) was added dropwise to a solution of 1-[amino(thioxo)methyl]-3,5-dimethyl-1*H*-pyrazole (**2**) (77.6 mg, 0.5 mmol) in ethyl acetate (5 mL) while stirring. The reaction mixture was stirred for 24 h. Obtained gray-green precipitate was filtered off, washed with ethyl acetate and diethyl ether and dried under reduced pressure. Yield: 128.0 mg (88.4%), and m.p: 164.8–166.2 °C. IR (KBr) ν (cm^{-1}): 3296, 3106 (NH_2), 1621, 1588 (C═C, C═N), 1346 (C – NH_2), 999 (N – N), and 847 (C═S). Anal. Calc. $C_6H_9N_3SCuCl_2$ (M = 289.675 g/mol) Anal (%): C 24.88, H 3.13, and N 14.51. Found (%): C 24.72, H 3.14, and N 14.48. LSI-MS (*m/z*): 289 ($LCuCl_2^+$); 254 ($LCuCl^+$); and 155 (L).

2.3. The stability of compounds in aqueous solution

The stability of the metal(II) complexes in water/DMSO solution at concentration 10 μM was assessed by UV–vis spectrophotometric analysis in the range of 200–800 nm. Spectra were recorded over a period of 24 h using a Scanning Kinetics program. The timetable of all measurements is presented in Table S1. The obtained UV–vis spectra were compared to each other.

2.4. Determination of partition coefficients (logP)

The lipophilicity of our compounds, i.e. their partition coefficient in an *n*-octanol/water system, was measured using the shake-flask method [30,31]. To suppress hydrolysis of the chloride ligands a 0.15 M NaCl buffer served as a water phase. The coefficients (log*P*) were expressed as the log10 of the ratio of the compound concentration in the *n*-octanol phase to the compound concentration in the aqueous phase. The mixture of the abovementioned solutions in ratio 1:1 was shaken mechanically for 24 h. All the measurements were executed only in n-octanol solution as none of tested compounds **3**–**5** was soluble in the used buffer. Then, the amount of compound in water phase was estimated as the difference between the absorbance of the compound in *n*-octanol and the absorbance of this sample after stirring it with water solution. The amount of compounds in *n*-octanol phase after stirring was determined by UV–vis spectroscopy on a Varian Bio 100 UV–vis spectrophotometer. The partition coefficients were expressed as the log10 of the ratio of the compound concentration in the *n*-octanol layer to the compound concentration in the aqueous layer. All measurements were executed at least seven times. Equal volumes (1 mL) of the aqueous solution and *n*-octanol were shaken together mechanically for 24 h.

2.5. CD absorption spectrum measurements

The CD spectra of all compounds were recorded in the range 200–500 nm (UV–vis) with a screening rate of 100 nm min^{-1} at room temperature. Double stranded DNA strand consisted of the following annealed strands: 5′-GGA GTA TGC TCA TTT CCA ATA CAT ACT CCT ATA GTG AGT CGT ATT AAT TTC-3′ and 5′-GAA ATT AAT ACG ACT CAC TAT AGG AGT ATG TAT TGG AAA TGA GCA TAC TCC-3′.

Compounds **3**–**5** (30 μM) were dissolved in a solution of DMSO/10 mM Tris–HCl, 50 mM NaCl and added to the double-stranded DNA (10 μM) in 10 mM Tris–HCl, 50 mM NaCl buffer in a molar ratio of nucleotide:compound = 30:1. A reference sample consisted of DNA. The data are expressed as the mean of Δε. All samples were incubated at room temperature for 24 h.

2.6. Cleavage of plasmid DNA

To check the influence of compounds **3**–**5** on DNA supercoiling, agarose gel electrophoresis was performed. Supercoiled plasmid DNA (2872 bp) in 10 mM Tris–HCl, 50 mM NaCl (pH 7.2) was incubated with our complexes (100 or 300 μM; DMSO/10 mM Tris–HCl, 50 mM NaCl) at 37 °C for 24 h. The samples were subsequently mixed with 1 μL of loading buffer (25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) to a final volume of 13 μL and loaded onto 1% (w/v) agarose gels including 2.5 μL of ethidium bromide. Electrophoresis was run for 1.5 h at 60 V in Tris–acetate–EDTA (TAE) buffer. The gels were analyzed by UV shadowing and the bands quantified by Image Quant TL (GE Healthcare).

2.7. Alkylating properties

Preussmann's test (NBP test) was performed to test the alkylating properties of our complexes. Each compound was dissolved in 10 μL of DMSO and 2-methoxyethanol added to a final volume of 1 mL (final concentration 100 μM). The equal volume of NBP (4-(*p*-nitrobenzyl)pyridine) in 2-methoxyethanol (5%) solution was added and the mixtures were heated to 100 °C for 1 h. After quick cooling to 20 °C, 1.25 mL methoxyethanol and 0.25 mL piperidine were added to give a total volume of 3.5 mL. After 90 s of incubation, the UV/vis absorbance at 560 nm was measured in the presence of 2-methoxyethanol (d = 1 cm).

2.8. Cytotoxic activity

2.8.1. Cell culture conditions and drug treatment

The cytotoxic activity of compounds **1**–**5** was tested against two head and neck squamous cell carcinoma (HNSCC) cell lines UMB-SCC-745 and UMB-SCC-969, melanoma A375 cells as well as human breast adenocarcinoma MCF7 and cervical cancer cells. Cells (100,000 cells/mL) were grown at 37 °C in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin (HNSCC, A375 and HeLa cells) or in Gibco MEM essential medium 1× (MCF7 cells), respectively. Compounds **1**–**5** were dissolved in DMSO and diluted with the growth medium RPMI 1640 or MEM (minimum essential medium), respectively, immediately before use. In every case, the final concentration of DMSO in the solution was smaller than 0.1%. Equivalent final concentration of DMSO was used in the control cultures.

2.8.2. Cytotoxicity assay on HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords as previously described [32], and cultured in plastic dishes coated with gelatin, in RPMI 1640 medium supplemented with 20% FBS (fetal bovine serum), 90 U/mL heparin, 150 μg/mL ECGF (endothelial cell growth factor, Roche Diagnostics, Mannheim, Germany) and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin). 10×10^3 cells were seeded on 96-well plates (Nunc) and exposed to compounds.

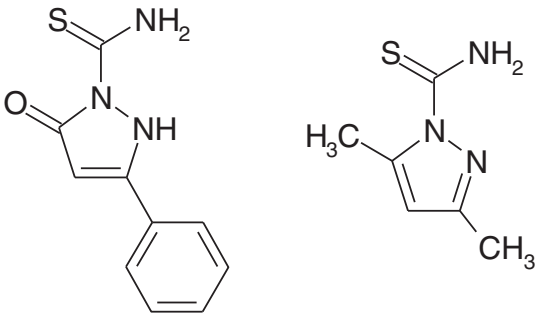
The values of IC_{50} (the concentration of test compound required to reduce the cell survival fraction to 50% of the control) were calculated from dose–response curves and used as a measure of cellular sensitivity to a given treatment.

2.8.3. Cell proliferation assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

The MTT assay was used to evaluate the influence of compounds **1**–**5** on cell proliferation of HNSCC, HeLa and MCF7 cells. Cells were cultured in 96-well plates for 12 h. Then, the cells were incubated with compounds **1**–**5** (in the final concentration 1–100 μM) for 2 days. MTT reagent (Sigma-Aldrich; 0.84 mg/mL in RPMI 1640 or MEM medium, respectively) was added and left in the cultures for 3 h at 37 °C. Next, 100 μL of DMSO (optical grade) was added to dissolve the insoluble blue formazan precipitate produced by reduction of MTT compound. The absorbance was determined at 565 nm by an ELISA (enzyme-linked immunosorbent assay) reader.

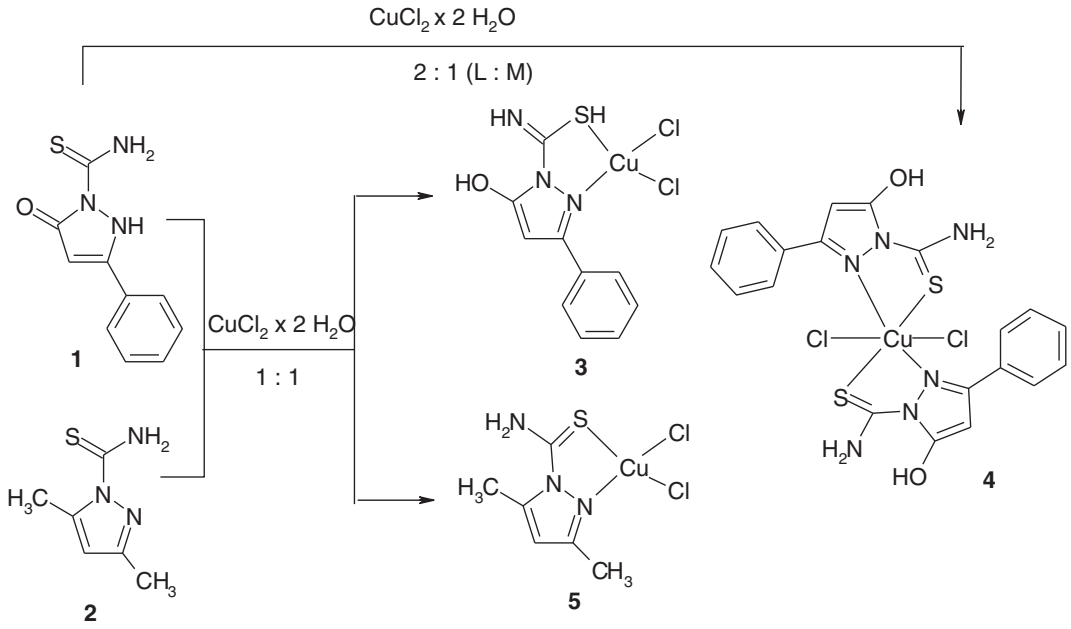
2.8.4. Cell proliferation assay: acid phosphatase activity (APA)

An inhibition of A375 cell proliferation by compounds **1**–**5** was examined by APA test. Cells were seeded in 24-well plates for 6 h. The cells were incubated with each compound (in the final concentration



1 **2**

Fig. 1. Structures of ligands **1** and **2**.



Scheme 1. Synthesis of copper(II) complexes with ligands **1** and **2**.

1–100 μM) for 2 days. Thereafter, cells were collected by centrifugation. APA reagent (sodium *p*-nitrophenyl phosphate/10 mL PBS/10 μL TRITON × 100; 13.16 mg) was added and left in the cultures for 2 h at 37 °C. Next, 40 μL 1 M NaCl was added to stop the reaction. The absorbance was determined spectrophotometrically at 450 nm using a microplate reader (Infinite M200 PRO, TECAN, Austria).

For each condition (MTT and APA assays), three assays were performed in at least four independent experiments. The mean of the absolute absorbance values given by drug-treated cells was divided by the mean of the absolute absorbance of DMSO treated control sample and expressed as relative number of viable adherent cells. All data given correspond to the mean of at least four independent experiments with one standard deviation. IC_{50} values were calculated from concentration–response curve fitting, using a Microsoft Excel-based analytic method.

2.9. Annexin V/propidium iodide/Hoechst staining

HeLa cells (7000 cells/mL) were incubated with compound **4** (2.5 μM) for 8, 24 and 44 h, respectively, to induce apoptosis or necrosis. Next, cells were washed with RPMI 1640 medium before the incubation buffer (1 mL, Roche), Annexin V (10 μL), propidium iodide (4 μL) and Hoechst (4 μL) were added. The cells were incubated with dyes for 15 min at room temperature. Then, the samples were examined, using confocal microscopy (CSLM SP5, Leica, magnification × 1000). In each experiment, more than 300 cells were analyzed and the percentages of early/late apoptotic or necrotic cells were calculated. The cells with bright blue chromatin of organized structure were counted as viable while cells with more intensive blue condensed chromatin and green membranes were counted as medium apoptotic. The cells with green fluorescence were counted as early apoptotic cells. In late apoptotic cells with green membranes, chromatin was condensed or fragmented and stained orange. Necrotic cells had bright orange chromatin with organized structure.

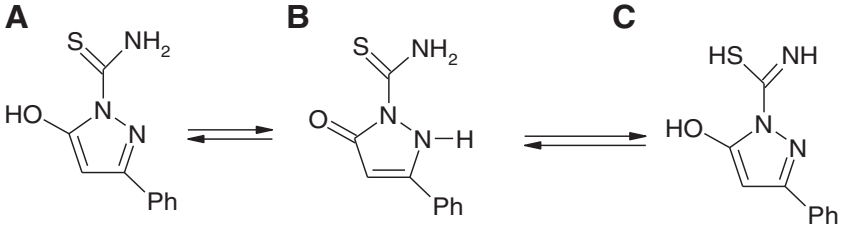
3. Results and discussion

3.1. Synthesis of compounds

For our study we have selected the two pyrazole derivates **1**, **2** as ligands (Fig. 1). Complexes **3** and **5** were prepared at room temperature in an ethyl acetate/methanol (9:1) mixture by adding equimolar amounts of copper(II) chloride and ligands **1** or **2**, respectively.

Complex **4** was prepared by mixing of $CuCl_2$ $2H_2O$ with 1-[amino(thioxo)methyl]-5-hydroxy-3-phenyl-1*H*-pyrazole (**1**) in 1:2 (M:L) molar ratio. The reaction was carried out in an ethyl acetate and methanol solution (4:1) and the mixture was refluxed for 2 h in 45 °C (Scheme 1).

The infrared spectra of all complexes **3**–**5** are similar. The structures of complexes **3** and **4** are assumed on the basis of their IR spectrum and comparison with the spectrum of ligand **1**. The strong bands in the spectrum of ligand **1** in the 3300–3100 cm^{-1} range are characteristic for the primary amide $\nu(NH_2)$ vibrations (3290 cm^{-1} NH_2 stretch, 3159 and 3113 cm^{-1} asymmetric and symmetric stretch, respectively). These bands are shifted to a lower frequency in complexes **3** and **4**. Ligand **2** exhibits bands due to $\nu(NH_2)$ moieties in the $\nu_{asym.}$ 3389 cm^{-1} and



Scheme 2. Tautomeric forms of ligand **1**.

$\nu_{sym.}$ 3241 cm^{-1}. These bands shift to a lower frequency at $\nu_{asym.}$ 3296 cm^{-1} and $\nu_{sym.}$ 3106 cm^{-1}.

The strong band at 1651 cm^{-1} for ligand **1** can be assigned to the ν(C═O) vibration, which vanishes for complex **3**. This band suggests that the ligand is present in its **B** tautomeric form, as is shown in Scheme 2. The band contributing to ν(C═S) at 880 cm^{-1} in the spectrum of ligand **1** shifts to a lower frequency in the spectrum of complex **3** and looses intensity. The new band at 2363 cm^{-1} for the ν(SH) vibration in complex **3**, supports the proposition on the **C** tautomeric form for ligand **1** in this complex. For complexes **4** and **5** the band at 880 cm^{-1} shifts to 867 cm^{-1} and 847 cm^{-1}, respectively, but exhibits the same intensity. The differing intensity and frequency of the bands in compounds **3** and **4** suggest that the sulfur atom participates in the coordination and that ligand **1** can appear as a **C** or **A** form, respectively (Scheme 2).

The most significant difference in the IR spectrum of ligand **1** compared to complexes **3**, **4** and **5** are observed for the N – N bands. These bands shift to lower frequency in all cases ($\Delta\nu$ = 61 cm^{-1}, $\Delta\nu$ = 25 cm^{-1} and $\Delta\nu$ = 30 cm^{-1} respectively), proving a direct coordination to the metal ion.

The ^{1}H NMR spectroscopy has been proven useful in establishing the structure and nature of many ligands and their diamagnetic complexes. All of our complexes are paramagnetic and therefore NMR spectroscopy does not always provide reliable results. We have made time-dependent NMR spectra for both ligands. The proton magnetic resonance spectrum of ligand **2** shows its existence in the enolic form. The NMR spectra were recorded in d_6-DMSO, and after 24 h we observed the appearance of a new peak at 2.8 ppm for the –SH group (Fig. S1).

Valuable structural information about the newly synthesized complexes **3**–**5** was obtained by mass spectrometric measurements. Parent peaks of the obtained complexes have been observed at m/z 353, 574 and 289 in MS spectra for complexes **3**–**5**, respectively. The ion [$LCuCl^+$] has been observed for compound **5** at m/z 254. Ion at m/z 281 has been observed for [LCu^+]. LSI-MS spectra of both ligands at m/z 219 (**1**) and 155 (**2**) presented ion peaks.

3.2. Physico-chemical properties

3.2.1. Stability of compounds

An important parameter of a potential drug is its thermodynamic stability over a prolonged period of time. All potential drugs should be stable under conditions of living organisms to reach their target. Complexes **3**–**5** were tested for their stabilities in water/DMSO solution (10 μM), containing 0.1% DMSO by means of UV–vis spectroscopy. The time-dependent (in the time course of 0, 12, 24 h) UV–vis spectra of each complex dissolved in water/DMSO solution are shown in Fig. 2. There were no obvious changes in the spectral characteristics and the peak absorptions for complexes **3** and **4** over the time, suggest no structural alterations in these complexes, but for complex **5** we have observed the decrease in the intensity of the UV–vis spectra and this change suggests that the stability of complex **5** is the lowest of all complexes in water/DMSO solution. The lack of stability of this complex can be linked to the ligand **2**, which is more unstable than the ligand **1** (see Fig. S11).

3.2.2. LogP

Transport into cell has a crucial influence on the biological activity of any drug. To track how molecules pass membranes or other barriers, one can use specific indicators such as the drugs' lipophilicity. Generally, molecules penetrate the cell membrane more easily with increasing lipophilicity because they interact better with the fatty acids of the lipid bilayers. However, if too lipophilic, the molecules will stick to the lipid bilayers and will not penetrate the membrane.

The lipophilicity can be described as log*P*, which is the decadic logarithm of the equilibrium concentration ratio of a compound in two immiscible phases. Generally, an octanol/water system is a suitable model

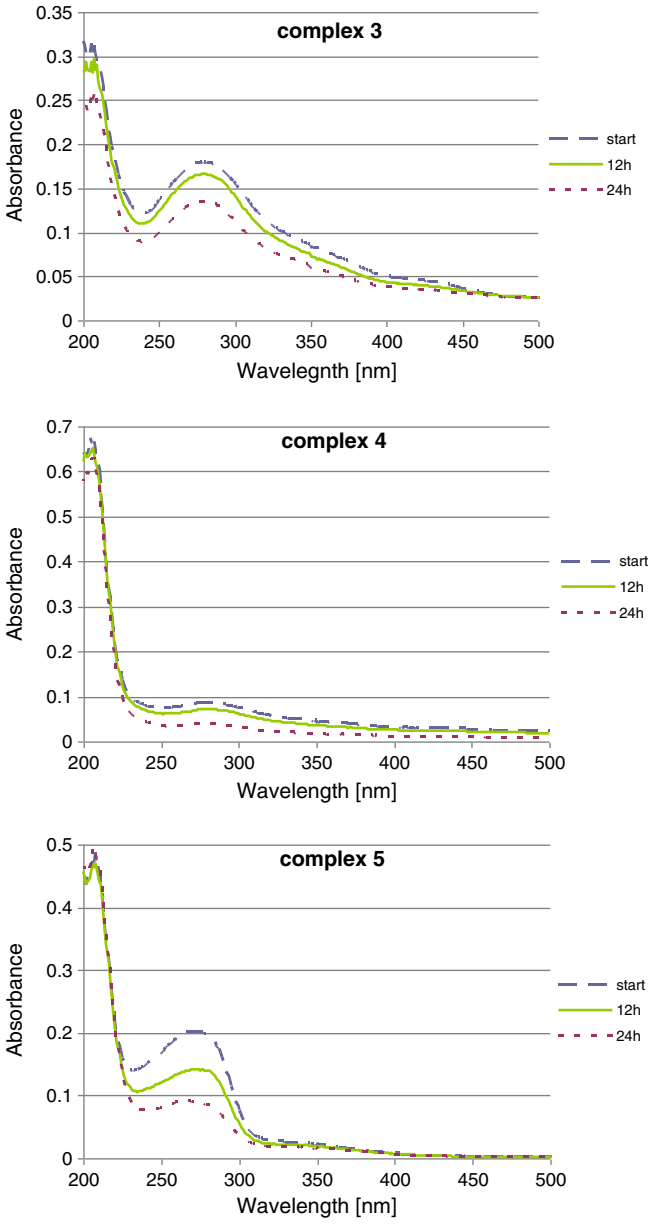


Fig. 2. Representative time-dependent UV–vis spectra of complexes **3**–**5** at 23 °C recorded at t = 0 (blue dashed line), t = 12 (green solid line), and t = 24 h (red dotted line) at the concentration of 10 μM.

to project an environment of drug transport in living organism as octanol possesses a similar polarity as lipid bilayers [33,34].

The lipophilicity for complexes **3**–**5** was determined by the so-called shake-flask method, a well established method to obtain log*P* values in the range −2.5 to 4.5 [35]. All measurements were carried out in 0.15 M NaCl buffer at pH 7.2 to ensure conditions similar to physiological

Table 1
Alkylating activity of compounds **3**–**5** determined by the Preussmann test. According to Preussmann: (−) A < 0.05, (+) A = 0.05–0.10, (++) A = 0.10–0.50, and (+++) A > 0.50 [50].

Compound	Preussmann value	Alkylating activity
3	0.05 ± 0.01	Very poor (+)
4	0.15 ± 0.02	Good (++)
5	0.1 ± 0.03	Good (++)
Cisplatin	0.30	Good (++) [57]
Carboplatin	0.20	Good (++) [57]

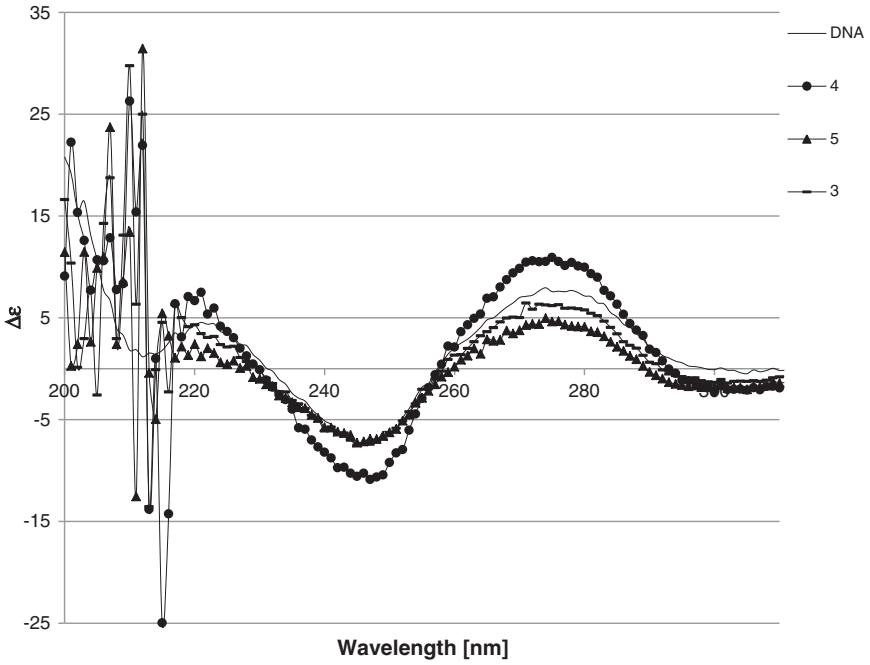


Fig. 3. Ellipticity of the DNA alone and in complex with compounds **3**–**5** in 10-fold excess.

conditions and to prevent possible hydrolysis of the coordinated chloride ions [36].

Compound **3** has a good lipophilicity with log $P = 1.01 \pm 0.07$, while compounds **4** and **5** are of lower lipophilicity with log*P* values of 0.27 ± 0.04 (**4**) and 0.49 ± 0.04 (**5**) (errors correspond to one standard deviation).

Since a compound is lipophilic if its log*P* value is higher than 0 we can conclude that complexes **3**, **4**, and **5** are all lipophilic and possess a more favorable lipophilicity than cisplatin, whose $\log P_{oct}$ value is around -2.28 ± 0.05 [25,37]. However, as the ability to cross membranes is a sum of many parameters, more advanced research is needed.

3.3. Biological activity

Many processes in living organisms are related to inheritance, e.g. gene expression, replication, DNA repair, or regulation, and rely on recognition of specific base pairs. Metal ions interact with nucleic acids not only to neutralize their charge, but also to exert strong influence on their structure and function [38–41]. Similarly, metal complexes are generally prone to interact with DNA in many different ways e.g. intercalation or groove binding. They can also lead to DNA alkylation and DNA strand cleavage. Moreover it is noteworthy that they possess a high affinity to DNA and are able to induce DNA breaks [42–44]. The mode of action of numerous anticancer drugs is related to their ability to interact with DNA or inhibit proper DNA relaxation. In fact, DNA is believed to be the main target of most anticancer drugs. Such compounds are able to destroy the DNA structure in cancer cell and lead to inhibition of cell growth as well as apoptosis [45–48].

3.3.1. Alkylating properties

The activity of many compounds commonly used in anticancer therapy is related to their alkylating property. Alkylation of nucleobases causes DNA breaks or stable crosslinks between DNA strands, with protein, or the drugs itself [49]. Covalent bonds of these types prevent the unraveling of nucleic acids, which is required in processes such as replication and transcription. If irreparable, these covalent bonds lead to apoptosis or necrosis of the cell [50,51]. Guanine *N*-7, adenine *N*-1 and *N*-3, as well as cytosine *N*-1 of DNA are the most frequent alkylated atoms [52].

Although cisplatin and carboplatin are not typical alkylating agents, these compounds can coordinate tightly to DNA by substituting the chloride ions [53–58].

The alkylating activity of complexes was determined with the Preussmann test using NBP [59].

The level of alkylation of NBP was measured by quantifying the absorbance at 560 nm.

The results shown in Table 1 were analyzed according to the Preussmann scale and show that complex **4** is the best alkylating agent of all tested compounds, with an absorbance value of 0.15. Compound **5** also possesses good alkylating activity, but its absorbance was 0.05 U lower when compared to complex **4**.

All tested compounds possess worse alkylating activity in comparison to cisplatin and carboplatin as their absorbance value was 0.30 and 0.20, respectively [60]. Lower activity of compounds **3**–**5** can be related to the differences in thermodynamic stability of platinum(II) and copper(II) compounds. Moreover, it is known that the nature of ligands can determine their alkylating properties. Ligands **1** and **2** did not indicate any alkylating activity as their absorbance was 0.

3.3.2. CD analysis

Complexes with planar ligands usually bind to DNA by stacking with DNA base pairs. The effectiveness of binding increases with the size and planarity of the ligands [61,62]. Intercalation may lead to an unraveling of DNA strands and their stiffening [63].

The influence of compounds **3**–**5** on the geometry of double stranded DNA was evaluated by CD spectra (for the sequence see Section 2). The CD spectra were recorded in 10 mM Tris–HCl, 50 mM NaCl buffer.

Table 2
Maximum ellipticity and wavelength of the CD signal of the dsDNA alone and incubated with compounds **3**–**5** at 10-fold excess, respectively.

	λ_{max}	Δε
DNA	274	7.95
3	271	6.46
4	275	10.92
5	274	4.97

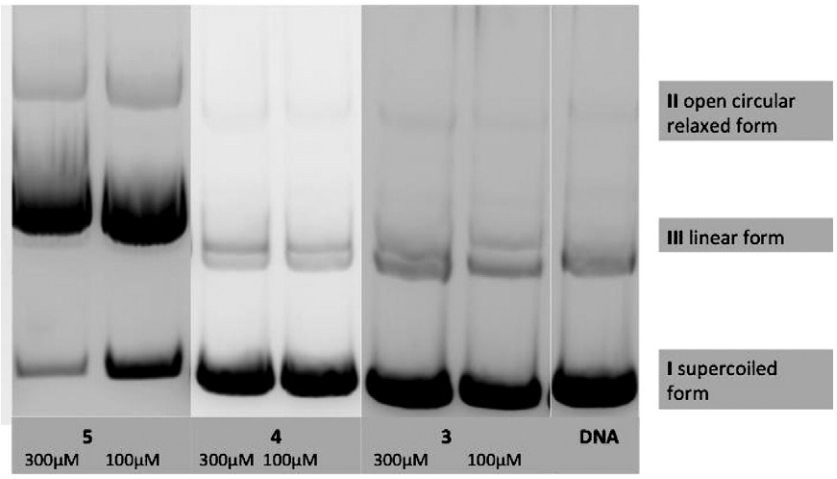


Fig. 4. Gel of plasmid DNA cleaved by compounds **3**–**5**.

In a first set of experiments we made sure that the addition of 1% of DMSO to the buffer has no influence on DNA stability and/or CD spectra.

Compounds **1**–**5** show no CD ellipticity between 260 and 280 nm, the spectral width to characterize DNA. In addition, complexes **3**–**5** only induce minor changes to the CD spectrum of the DNA (Fig. 3). The λ_{max} value of the positive band shifted by less than 3 nm for compound **3** (Table 2) and the Δε value is lowered by 1.49 U. The lowest Δε value was obtained for compound **5** (Δε = 4.97), which shows no shift of the λ_{max} value. Our results suggest that all tested compounds have only moderate influence on the DNA structure. Only for compound **4**, Δε shifts by about 2.97, which could be related to the relaxation of dsDNA by this compound.

*3.3.3. Electrophoretic mobility of plasmid DNA in the presence of **3**–**5***

The biological activity of drugs is often related to their ability to cleave DNA. They are able to bind to DNA either specifically or sequence-independent and cleave one or both strands by either a radical or a hydrolytic pathway, the latter similar to the one of natural nucleases [64–68].

The cleavage of plasmid DNA (2872 bp) by compounds **3**–**5** was monitored by agarose gel electrophoresis. Strand cleavage of the naturally occurring supercoiled DNA (Form I) led either to an open circular relaxed form (Form II) upon single strand cleavage or to a linear form (Form III) upon double strand cleavage. The different forms could be distinguished by agarose gel electrophoresis, as form I migrated faster than the other forms, while form II was the slowest of the three due to its relaxed structure (Fig. 4) [69,70].

To prevent DNA aggregation, the final concentration of **3**–**5** in each experiment was never higher than 300 μM. The results presented in Fig. 4 show that only compound **5** was able to cut dsDNA under applied conditions.

Quantification of the gel confirmed that increasing concentration of compound **5** caused a reduction in band intensity of I. The obtained results also show that this compound cut supercoiled DNA to linear form III with quite similar efficacy in both tested concentrations. Moreover, compound **5** had moderate influence on the amount of form II. In conclusion, only complex **5** cleaved plasmid DNA effectively (Fig. 5).

3.3.4. Cytotoxic activity

The cytotoxic effect of anticancer drugs might be specific to particular types of cancers. Therefore, the activity of compounds **1**–**5** was tested against four different cancer cell lines: human HNSCC (SCC-745 and SCC-969), HeLa, A375 and MCF7.

The influence of compounds **1**–**5** on the metabolic activity of HNSCC, HeLa and MCF7 cells in relation to untreated cells was assessed by MTT assays, the cytotoxic activity against the A375 cell line by an APA test. The incubation time was two days and the concentration–response analyses were done from 0.5 μM to 200 μM. The cytotoxicity of any compound is usually expressed as the concentration that reduces the cell viability by 50% in relation to untreated cells. These IC_{50} values obtained for each particular cell line are summarized in Table 3. Melanoma A375 was the most sensitive cell line for all compounds with IC_{50} values ranging from 2 μM to 5 μM. It is an interesting result as in our previous study, this melanoma cell line was highly resistant to 5 μM cisplatin [74].

Complex **4** was the most cytotoxic compound against A375, MCF7 and HeLa cell lines with IC_{50} values being 8.1, 7.2 and 6.5 times lower than cisplatin. Complex **4** was also able to inhibit the proliferation of HNSCC 969 and HNSCC 745, but was less efficient than cisplatin. The other two complexes possessed very weak cytotoxic activity against HNSCC 969, HNSCC 745, HeLa and MCF-7 cell lines with IC_{50} values >100 μM.

Cytotoxicity of ligands **1** and **2** (even at concentrations of 200 μM) was very weak against all tested cell lines as they reduced cell proliferation only to about 87–90% when compared to untreated cells (data not shown). It is known from the literature that cytotoxicity of $CuCl_2$ and $Cu(ClO_4)_2$ against cancer cells is low [75,76]. Thus, we conclude that cytotoxicity was generated rather by the complexes than the ligands. Unfortunately, the cytotoxicity of complex **4** on HUVEC cells was similar to the cytotoxicity of cancer cells with IC_{50} values 2.00 ± 0.10. However, it is known from literature that HUVECs are often used to investigate anti-angiogenic effects [77], and therefore it is possible that compound **4** could be of interest for clinical trials.

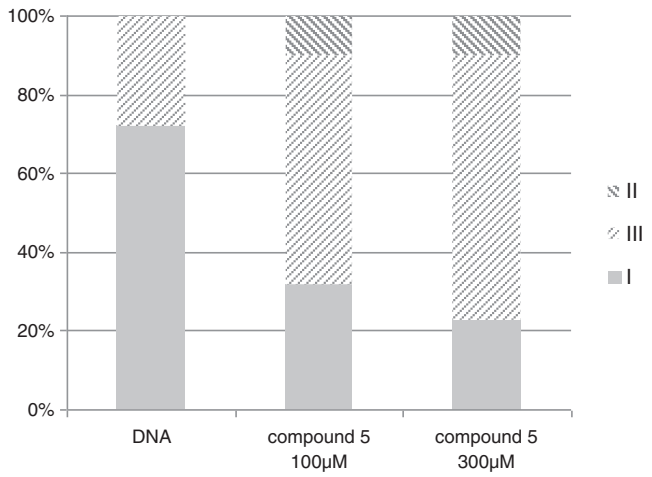


Fig. 5. Quantification of plasmid DNA incubated with complex **5**.

Table 3
IC_{50} values for compounds **3**–**5** against all used cell lines. The data are the average of three independent experiments done in triplicates. Bolded results mean higher activity than cisplatin.

	HNSCC 969	HNSCC 745	MCF7	HeLa	A375
3	>200 μM	>200 μM	>200 μM	>200 μM	**2.5 ± 0.4** μM
4	49.7 ± 1.0 μM	18.3 ± 0.8 μM	**1.8 ± 0.4** μM	**2.5 ± 0.2** μM	**5.0 ± 0.8** μM
5	>200 μM	>200 μM	>200 μM	>200 μM	**2.0 ± 0.8** μM
Cisplatin	5 μM	2.5 μM	13.0 μM [71]	16.3 μM [72]	20.3 μM [73]

3.3.5. Analysis of apoptotic cells

Observations that apoptosis in cancer cells can be spontaneous, inspired scientists to broaden their research spectrum of potential anticancer drugs to compounds that are able to induce programmed cell death [78]. For example, the cytotoxicity of cisplatin can be a consequence of its ability to activate signal transduction pathways in cancer cells, which are related to DNA damage [79,80].

One typical feature of early apoptosis is the loss of symmetry of the cell membrane phospholipids. This leads to the displacement of phosphatidylserine within the cell and subsequent leakage. This process can be useful to discern early apoptotic cells by Annexin V staining, which yields, due to its high affinity towards phosphatidylserine, green fluorescence upon. Annexin V (AnnV) may also bind to phosphatidylserine on late apoptotic and necrotic cells but as these cells lose their membrane integrity, they can be distinguished from early apoptotic cells by the usage of propidium iodide (PI). Propidium iodide is also able to penetrate into dead cells, giving red fluorescence. Hoechst staining, on the other hand, gives a blue fluorescence in normal cells.

The ability of compound **4** to induce apoptosis in cancer cells was assessed by investigating apoptotic cells in control cells as well as in cells treated with compound 4 for 8, 24 and 44 h (Fig. 6). HeLa cells were chosen for this assay as compound **4** exerted the highest activity in this cell line, in relation to activity of cisplatin. In untreated cells, only 1% of apoptotic and 1.25% of necrotic cells were observed after 44 h. In cells treated with complex **4**, no significant numbers of apoptotic and necrotic cells were observed after 8 h. 5% apoptotic cells were observed after 24 h, and the percentage increased to 50% (30% of early apoptotic and 20% of late apoptotic cells) after 44 h. Longer incubation with compound **4** did not cause an increase in the amount of necrotic cells. Evidently, compound **4** is an effective inductor of apoptosis in HeLa cells. However, to understand its mode of action, more advanced experiments are needed.

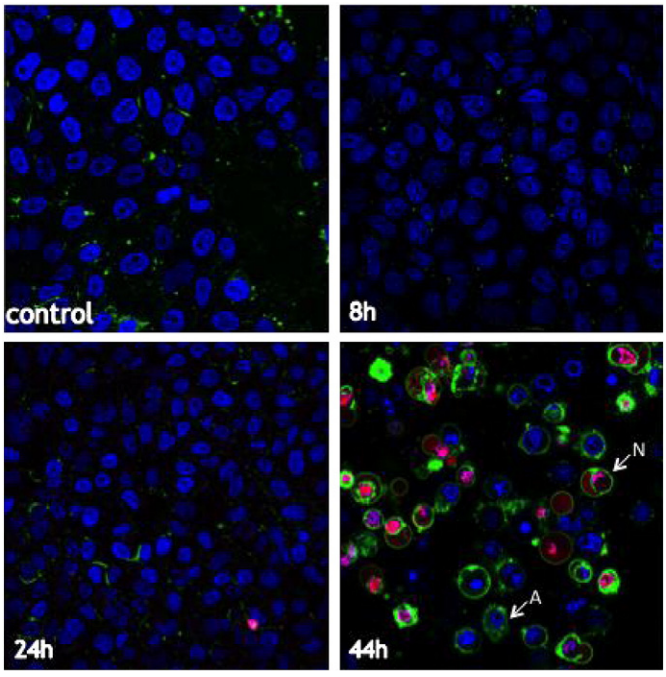


Fig. 6. Cancer cell response to the treatment with compound 4 visualized as the morphological changes that occur during apoptosis. Confocal microscope (magnification ×1000) was used to assess induction of apoptosis in HeLa cells after 8 h, 24 h and 44 h of treatment. Intact cells are stained exclusively with Hoechst (blue fluorescence). In early apoptotic cells, marked with "A", only cellular membrane is stained with Annexin V (green fluorescence). Late apoptotic cells are stained with Annexin V (green fluorescence) and PI (pink fluorescence). Some cells, considered as necrotic, are stained mainly with PI (marked with "N").

4. Conclusion

Two ligands and three different complexes thereof with Cu(II) were prepared and characterized in detail. UV–vis spectra recorded at different intervals within 44 h indicated that all complexes are stable in aqueous solution. Complexes **3**–**5** have positive log*P* values, suggesting that they are all lipophilic, with compound **3** being the most promising with regard to cell membrane penetration. Preussmann's tests showed that complexes **4** and **5** are rather good "alkylators", suggesting that both compounds could effectively react with DNA bases. None of the two ligands indicated any alkylating activity, showing that the alkylating properties of the complexes **3**–**5** are intrinsic. All complexes were, however, less active than cisplatin, whose value is twice as high as that of the most effective compound (**4**).

CD spectrum analysis indicates that only compound **4** alters the DNA structure as it causes the ellipticity to higher values. It is possible that compound **4** can behave as DNA intercalator. Complex **5**, on the other hand, is able to modify the structure of plasmid DNA under both tested concentrations by nicking the DNA form I into forms II and III. The effectiveness of this process depends on the concentration. Upon increasing amounts of complex **5**, form I of plasmid DNA diminishes whereas forms II and especially III accumulate. Additional analysis (executed by using Image Quant program) confirmed the obtained results. It is possible that the ability to cleave the DNA is the predominant mechanism for the biological activity of compound **5** against A375 cells.

Complexes **3**–**5** showed cytotoxicity against four cancer cell lines. All three complexes were more cytotoxic to A375 cells in comparison to cisplatin. Compound **4** was cytotoxic towards all tested cell lines, but it was particularly active against MCF7, A375 and HeLa cells and, interestingly, at lower concentration than cisplatin. As both ligands were not cytotoxic against the tested cell lines, the activity of complexes **3**–**5** was obviously an intrinsic property of the complexes and not due to the ligand alone. Moreover, compound **4** was a very good inductor of apoptosis in HeLa cells, and its effectiveness was increased with longer incubation. After 44 h of incubation, the percentage of apoptotic cells was increased to 50%. By contrast, the presence of complex **4** did not lead to an increase of necrosis in HeLa cells. It can thus be assumed that apoptosis is the major mechanism of its anticancer activity.

Our results show that compounds **3** and **5** are toxic only against particular types of cancer. The most promising is compound **4** as this complex shows high cytotoxicity against all tested cell lines, a favorable lipophilicity and the ability to induce programmed cell death. Consequently, this is a very good basis for continued research towards the optimization of this complex by synthesizing derivatives thereof to obtain even higher specificity towards cancer.

We have now also investigated the cytotoxic effect of the most active complex **4** on HUVEC but it appeared that IC_{50} is similar for HUVEC as for cancer cells.

Abbreviations

LSI-MS	liquid secondary ion mass spectrometry
IR	infrared
NBP	4-(*p*-nitrobenzyl)pyridine
HNSCC	head and neck squamous cell carcinoma
HeLa	cervical cancer cells
ECGF	endothelial cell growth factor
HUVEC	human umbilical vein endothelial cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
APA	acid phosphatase activity
log*P*	partition coefficients
CD	circular dichroism
NBA	3-nitrobenzylalcohol

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2014.02.014.

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