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Antiproliferative and proapoptotic activity of molecular copper(II) complex of *N*-1-tosylcytosine

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ABSTRACT

In an attempt to enhance the previously observed antiproliferative capacity of 1-(*p*-toluenesulfonyl)cytosine (*N*-1-tosylcytosine, ligand **1**), its copper(II) complex ($Cu(1\text{-}TsC\text{-}N3)_2Cl_2$, complex **2**) was prepared and tested *in vitro* on various carcinoma and leukemia cells. The comparative *in vitro* studies using the ligand **1**, the complex **2**, $CuCl_2$ x $2H_2O$ salt (salt **3**) and the 1:2 mixture of the salt **3** and ligand **1** (mixture **4**) were performed on normal (WI38), human carcinoma (HeLa, CaCo2, MiaPaCa2, SW620), lymphoma (Raji) and leukemia (K562) cell lines. Significantly elevated concentration of the intracellular copper after treatment of K562 cells and HeLa cells during 2 h with complex **2** (7.83 *vs.* 5.4 times) was detected by atomic absorption spectroscopy. Cytotoxicity was analyzed by MTT assay. We found that antiproliferative capacity of the tested compounds varies (IC_{50} after 72 h of exposure: 0.6×10^{-6} M to $> 100 \times 10^{-6}$ M). Leukemia and lymphoma cells were found the most sensitive to complex **2** which showed more than 100 times higher *in vitro* activity against K562 cells than ligand **1**. Apoptotic morphological changes, an externalization of phosphatydilserine, and changes in the mitochondrial membrane potential of treated cells were found. The caspase-3 activity in HeLa and K562 cells was measured by caspase-3 colorimetric assay kit. Caspase-3 was not activated in the treated K562 cells while salt **3** and the mixture **4** in the HeLa cells significantly increased tested enzyme activity. These findings suggest that copper(II) in the molecular complex **2** by improving entry of the *N*-1-tosylcytosine **1** into cells increases its antiproliferative capacity.

In summary, the present study demonstrated that complex **2** possesses an antileukemic effect on K562 cells, and its anticancer activity was attributed with induction of apoptosis. The exact mechanism of apoptosis induction by complex **2** must be further investigated.

1. Introduction

Sulfonamides are very useful pharmaceutical compounds exhibiting a wide range of biological activities such as antibacterial [1,2], anticancer and antiviral [3,4], carbonic anhydrase inhibitory [5,6], and anti-inflammatory activity [7]. Also, a large number of structurally diverse sulfonamide derivatives have been studied as candidates for development of anticancer drugs [8,9]. It has been reported that the biological activity of some sulfur containing compounds could be enhanced by coordination with metals [10,11]. Metal-chelates of some sulfonamide derivatives have been found to exhibit stronger bacteriostatic activity than the ligands themselves [12]. Generally the use of metal complexes as new chemotherapeutics or diagnostic agents has rapidly increased in recent times [13–15]. In the anticancer research, high attention is paid to non-platinum metal complexes such as those of ruthenium, gold, copper and silver, which showed strong *in vitro* anticancer activity and also overcome negative side-effects encountered with cisplatinum and other platinum-based anticancer drugs [16,17]. Highly important role of copper in many biological processes including metalloenzymes, redox related transformations [18] and anti-inflammatory effects [19] inspired many investigations on Cu(II) complexes as potential anticancer agents [20,21].

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We have reported on the synthesis of novel pyrimidine nucleobase derivatives containing a sulfonamide pharmacophore [22–24]. The compounds showed strong antitumor activity *in vitro* [25–27] and *in vivo* [28–30] conditions. This type of nucleic base derivatives was found to inhibit DNA, RNA, and protein syntheses and induce apoptosis in human tumor cells [30,31]. Encouraged by the numerous reports on anticancer activity of complexes, we examined the possibility to prepare 1-(*p*-toluenesulfonyl)cytosine (ligand **1**) metal complexes and assess their biological activity. We have shown that ligand **1** is capable to form palladium (II) [32] and other transition metal complexes including Cu(II) and provided the X-ray crystallographic evidence of their structure [33].

In this study the results of *in vitro* cytotoxicity screening and proapoptotic potential of free ligand **1**, complex **2**, salt **3** and the mixture **4** against human tumor cells are reported.

2. Experimental

2.1. Synthesis

2.1.1. Preparation of the ligand *1* (1-TsC)

Synthesis of 1-(*p*-toluenesulfonyl)cytosine (ligand **1)** was performed according to the procedure reported by Kašnar-Šamprec et al. [28].

A mixture of cytosine (1 mmol) and *N,O*-bis(trimethylsilyl)acetamide (BSA) (3 mmol) was heated under reflux in dry acetonitrile (3.3 mL) for 1 h. The solution was cooled to 0 °C and tosyl chloride (1.2 mmol) was added. The reaction mixture was heated for 16 h at 80 °C, cooled and treated with a small amount of methanol. The resulting solid was filtered off and recrystallized from methanol yielding *N*-1-tosylcytosine **1** (80%) as a white crystals: ^{1}H NMR (DMSO-d_6) δ/ppm: 8.14 (d, 1H, $J_{6,5}$ = 7.8 Hz, H-6), 7.95 (brs, 2H, NH_2), 7.87 (d, 2H, J = 8.1 Hz, Ph), 7.46 (d, 2H, J = 8.1 Hz, Ph), 5.98 (d, 1H, $J_{5,6}$ = 7.8 Hz, H-5), 2.42 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ/ppm: 166.27 (s, C-4), 151.22 (s, C-2), 145.61 (s, Ph), 139.73 (d, C-6), 134.47 (s, Ph), 129.80 (d, Ph), 129.02 (d, Ph), 97.50 (d, C-5), 21.20 (q, CH_3).

2.1.2. Preparation of the Cu(1-TsC-N3)$_2$Cl$_2$ complex *2*

Synthesis of Cu(1-TsC-*N*3)$_2$Cl$_2$ (complex **2**) was performed by modifying our method [33] from more concentrated methanol solution.

To a warmed solution of *N*-1-tosylcytosine **1** (245 mg, 0.92 mmol) in methanol (20 mL) a hot solution of $CuCl_2$ x $2H_2O$ salt (78 mg, 0.46 mmol) in methanol (9 mL) was added. By cooling the solution precipitation of blue-green crystals occurred. The solid was filtered off and washed with a small amount of cold MeOH yielding 230 mg (75%) of complex **2**: ^{1}H NMR (DMSO-d_6) δ/ppm: 8.25 (brs, 1H, H-6), 7.89 (brs, 2.5H, Ph + part of NH_2), 7.42 (brs, 3.5H, Ph + part of NH_2), 2.34 (brs, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ/ppm: 143.22 (s, Ph), 132.13 (s, Ph), 127.58 (d, Ph), 126.75 (d, Ph), 19.27 (q, CH_3).

2.2. Biological studies

2.2.1. Cell lines and cell culture conditions

All cell lines used in this work were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and preserved in a liquid nitrogen prior use. Cells were tested and authenticated using the short tandem repeat profiling (STR) analysis.

Human fibroblasts (WI38) and tumor cell lines: pancreatic carcinoma (MiaPaCa2), colon adenocarcinoma (CaCo-2), cervix adenocarcinoma (HeLa), and metastatic colon carcinoma cells (SW620) were grown in a monolayer and cultured in the Dulbecco's modified Eagle medium – DMEM (Gibco, EU) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, EU), 2 mM glutamine, and 100U/0.1 mg penicillin/streptomycin. To detach from the flask surface, cells were trypsinized using 0.25% trypsin/EDTA solution. Raji (lymphoblastoid cells derived from Burkitt lymphoma) and K562 (erythromyeloblastoid leukemia cells**)** cell lines cultivation were grown in the RPMI 1640 medium (Gibco, EU) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, EU), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 100U/0.1 mg penicillin/streptomycin was used. Cells were cultured in a humidified atmosphere at 37 °C, 5% of CO_2 in the CO_2 incubator (IGO 150 CELLlife™, JOUAN, Thermo Fisher Scientific, Waltham, MA, USA). The trypan blue dye exclusion method was used to assess cell viability.

2.2.2. Cytotoxicity evaluation by MTT assay [34]

Cytotoxic effects of ligand **1**, complex **2**, salt **3** and the mixture **4** were assessed by MTT assay, carried out in 96-well microtiter plates (Greiner, Frickenhausen, Austria). The adherent cells (WI38, CaCo2, HeLa, MiaPaCa2, and SW620) were seeded at a concentration of 2×10^4 cells/mL and left overnight in the CO_2 incubator allowing them to attach to the plate surface, while Raji and K562 where seeded into 96-well plates immediately before compounds addition. Stock solutions of ligand **1**, complex **2**, the mixture **4** were made in DMSO and salt **3** in 10% DMSO, and used for preparation of working dilutions prior to addition to cells. Final concentration range was from 10^{-7} M up to 10^{-3} M. After expired time of incubation (72 h), growing medium was discarded and 5 mg/mL of MTT solution was added to each well, and incubated at 37 °C for 4 h. The reaction was determined by dissolving MTT-formazan crystals in DMSO. Absorbance was measured at 570 nm on Elisa micro plate reader (iMark, BIO RAD, Hercules, CA, USA). Control non-treated cells were grown under the same conditions. All experiments were performed at least three times in triplicates. The IC_{50} value, defined as compound concentration leading to cellular viability reduction by 50%, was calculated.

Percent of live cells was calculated as follows:

$$\% = \frac{\text{OD sample} - \text{OD backgroud}}{\text{OD control} - \text{OD background}} \times 100$$

2.2.3. Measurement of the intracellular copper concentration

The K562 and HeLa cells were plated into six well plates at a density of 8×10^6 cells in the cell culture medium and incubated in presence or absence of 1×10^{-3} M of ligand **1**, complex **2**, salt **3** and the mixture **4** respectively for 2 h in the CO_2 incubator. Cells were then collected in a tube and centrifuged for 5 min at 1100 rpm. Obtained supernatant was carefully removed and stored until analysis. Obtained pellet was washed three times with 10 mL PBS, centrifuged, and supernatant was carefully removed from the cells. Subsequently, on the cells were added 500 μL of sterile H_2O and after vortexing cells were frozen in liquid nitrogen and thawed in the warm water. The process was repeated 5 times. Cells were then centrifuged at 14,000 rpm and obtained samples were stored at −20 °C until analysis. Determination of intracellular copper in lysates of HeLa and K562 cells and in the growth medium were done by atomic absorption spectroscopy in an analytical system Agilent AA FS240. For calibration were used a commercial calibrator RECIPE ClinChem serum calibrator for trace elements. Calibration is performed in four points ($r > 0.99$). To check the operation of the analytical system and accuracy of calibration were used commercial control samples RECIPE ClinChek serum control for trace elements at two concentration levels. The measured values of copper in the control samples are very close to the target values set by the manufacturer of the control material), thus confirming the precision and accuracy of measurements. Experiments were done at least in triplicate and represent independent replicates experiments.

2.2.4. Determination of apoptosis induction by flow cytometry

Based on cytotoxic results obtained by MTT test (Table 1), proapoptotic potential of investigated compounds ligand **1**, complex **2**, salt **3,** and the mixture **4** were tested on HeLa and K562 cells using Annexin V-FITC and propidium iodide dye (Annexin V-FITC Apoptosis Detection Kit; Abcam, UK, EU). Cells were plated in 6-well plates at a

Table 1
Sensitivity of human tumor and normal cells towards investigated compounds.

Compounds	IC_{50} (10^{-6} M)						
	Normal cells	Leukemia cells		Carcinoma cells			
	WI38	K562	Raji	HeLa	MIAPaCa2	CaCo2	SW620
ligand **1**	> 100	80 ± 3.1	> 100	> 100	90.1 ± 11.1	95.0 ± 8.3	> 100
complex **2**	90.1 ± 7.7	0.6 ± 0.1	7.0 ± 0.3	10 ± 0.7	24.0 ± 0.2	20.0 ± 6.0	54.8 ± 0.9
salt **3**	93.0 ± 0.2	0.7 ± 0.2	8.4 ± 1.5	9.4 ± 0.5	19.0 ± 0.14	10.5 ± 0.1	4.0 ± 0.3
mixture **4**	> 100	56.6 ± 6.1	73.0 ± 0.8	> 100	> 100	> 100	87.1 ± 1.6

IC_{50}–Drug concentration that inhibited cell growth by 50%. Data represents mean IC_{50} (μM) values ± standard deviation (SD) of three independent experiments. Exponentially growing cells were treated with compounds during 72 h. Cytotoxicity was analyzed using MTT survival assay.

concentration of 3×10^5 cells/well for HeLa cells and 5×10^5 cells per well for K562 cells. After 24 h cells were treated with tested compounds **1–4** at the concentration of 10^{-5} M. After treatment cells were centrifuged at 3000 rpm for 5 min, stained according to the manufacturer's protocol, and analysed by flow cytometry (FacsCanto II, BD Biosciences, USA) using Flowlogic software (Inivai Technologies, USA).

2.2.5. Caspase-3 activity assay

Caspase-3 activity was measured according to the manufacturer's instructions (Caspase 3 Assay Kit, Colorimetric, Sigma-Aldrich, EU). HeLa and K562 cells were seeded at concentration of 3×10^5 cells per well in six well plates and treated for 24 h with tested 10^{-5} M compounds **1–4**. Cells were then lysed with 100 μL of chilled cell lysis buffer on ice for 15–20 min. After centrifugation (16,000 × *g*, 10–15 min, 4 °C), the supernatant was transferred in a new tube and analyzed at 405 nm using the ELISA reader (iMark, BIO RAD, Hercules, USA). Caspase 3 activity is expressed as μmol of released *p*-nitroaniline (pNA) per min per mL of cells' lysate.

2.2.6. Measurement of mitochondrial membrane potential (ΔYm) by flow cytometry

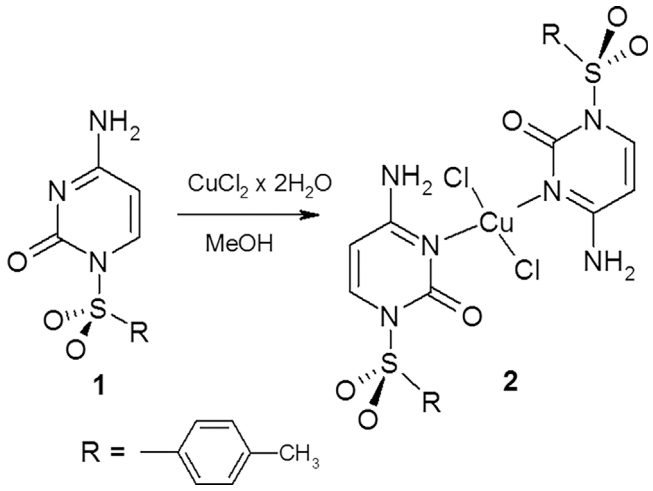
Changes in the mitochondrial membrane potential (ΔΨm) of treated cells were measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) dye. Briefly, tested cells were plated in 6-well plates at a concentration of 3×10^5 HeLa cells per well and 1.5×10^5 K562 cells per well, and incubated for 24 h with 10^{-5} M ligand **1**, complex **2**, salt **3** and the mixture **4**, respectively. After treatment, cells were centrifuged at 600 × *g* for 4 min and stained according to the manufacturer's protocol (Mitochondrial Membrane Potential Kit, Sigma-Aldrich, EU). Changes in the mitochondrial membrane potential were measured by flow cytometry (FacsCanto II, BD Biosciences, USA) and analyzed using Flowlogic software (Inivai Technologies, USA).

3. Results

3.1. Synthesis

1-(*p*-Toluenesulfonyl)cytosine (ligand **1**) was synthesized by condensation of silylated cytosine with *p*-toluenesulfonyl chloride as described previously [28]. The molecular complex Cu(1-TsC-*N*3)$_2$Cl$_2$ **2** was prepared in 75% yield by modifying our method [33] in the reaction of Cu(II) chloride with the ligand **1** in a 1:2 molar ratio of reactants and from the concentrated methanol solution (Scheme 1).

The ligand **1** and complex **2** are stable, non-hygroscopic, insoluble in most organic solvent, but soluble in DMSO and DMSO/H_2O mixture. ^{1}H NMR study [33] and mass spectrometry (see Fig. S1 in Supplemental materials) support the stability and formation of the above compounds.



Scheme 1. Synthesis of Cu(1-TsC-*N*3)$_2$Cl$_2$ (complex **2**).

3.2. Cytotoxicity

Cytotoxic potential of the ligand **1**, molecular complex **2**, salt **3** and the mixture **4** was tested on normal human fibroblasts (WI38), tumor cell lines growing in a monolayer, such as pancreatic carcinoma (MiaPaCa2), colon adenocarcinoma (CaCo-2), cervix adenocarcinoma (HeLa), and metastatic colon carcinoma cells (SW620), as well as on Burkitt lymphoma (Raji) and erythromyeloblastoid leukemia cells (K562) after 72 h of testing.

As shown in Table 1, cytotoxic potential of tested compounds depended on treated cells' type. Complex **2** displayed 50% growth inhibition of carcinoma cells in the concentration range 4.0–20.0 × 10^{-6} M. Leukemia and lymphoma cells were found to be the most sensitive and 50% of K562 cells growth inhibition was observed when 0.6 × 10^{-6} M complex **2** and 0.7 × 10^{-6} M of salt **3**, respectively, were applied. Ligand **1** was less efficient and showed similar inhibitory potential against normal and tumor cells.

3.3. Intracellular copper concentration

Intracellular copper concentration was significantly increased in treated K562 cells after 2 h of incubation with tested compounds compared to control cells (Fig. 1).

Compared with nontreated cells, substantially higher concentration of intracellular copper (by 7.83 times) were detected in by complex **2** treated cells, while in the cells treated by salt **3** the copper concentration increased by 4.37 times. A less increase in the intracellular copper concentration (by 1.85 times) was measured in the K562 cells treated by the mixture **4**. In the HeLa cells treated with complex **2** significantly increased concentrations of copper (by 5.4 times) were observed in comparison with control cells. In the HeLa cells treated by salt **3**

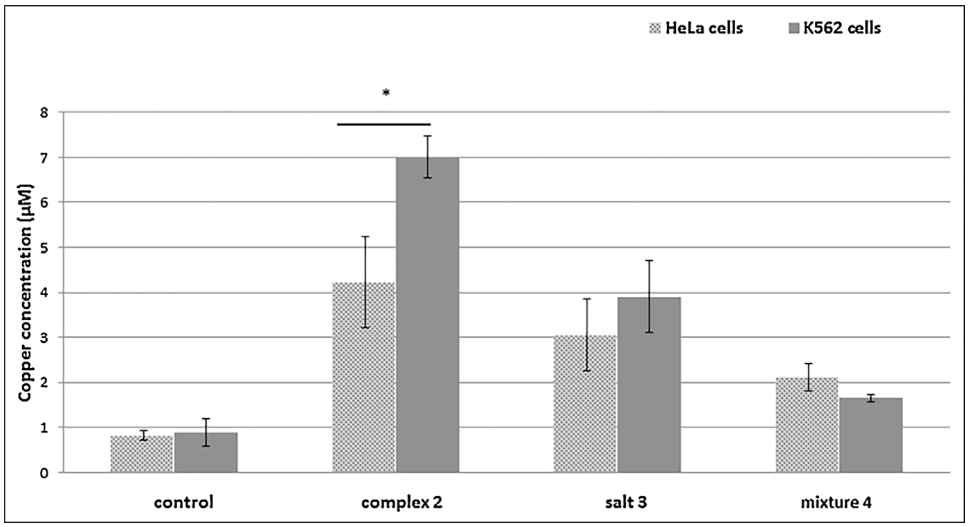


Fig. 1. Intracellular copper concentration in treated K562 and HeLa cells and nontreated (control) cells after 2 h of incubation with 1×10^{-3} M compounds detected by atomic absorption spectroscopy. Data represents mean values ± standard deviation (SD) of three independent experiments. *Statistically significant, $p < 0.05$.

intracellular copper concentrations increased by 4.08 times and in HeLa cells treated by the mixture **4** for 2.6 times. A statistically significant difference between copper concentration in the K562 and HeLa cells (7.83 times *vs.* 5.4 times) treated by complex **2** was observed.

3.4. Proapoptotic potential of tested compounds

3.4.1. Phosphatidylserine externalization

For assessment of apoptosis, we examined the exposure of phosphatidylserine on the cell surface using Annexin-V/PI double staining by flow cytometry. A capability of ligand **1** to induce apoptotic cell death was detected in our previous studies [25]. Our current results show that exposure of HeLa cells to tested compounds **1–4** ($c = 1 \times 10^{-5}$ M) during 24 h treatment slightly increased a number of necrotic cells (Fig. 2A, quadrant Q1) while number of apoptotic cells remained almost the same (Fig. 2A and B) in comparison with control non treated cells. All investigated compounds significantly induced externalization of phosphatidylserine at the outer membrane surface as a universal process occurring during early apoptosis in K562 cells (Fig. 2A and B). Necrotic features had 2–3% of treated K562 cells only (Fig. 2A, quadrant Q1).

3.4.2. Detection of the caspase-3 activity

Cells, HeLa and K562, were treated with tested compounds for 24 h. Obtained results, shown in Fig. 2C, indicate different answer of treated cells on applied compounds **1–4**. While in the treated leukemia cells caspase-3 activity, expressed as μmol of released *p*-nitroaniline (*p*NA) per min per mL of cells' lysate, was similar as in control cells, in the HeLa cells salt **3** and the mixture **4** increased enzyme activity for 40% compared to nontreated control cells.

3.4.3. Changes in the mitochondrial membrane potential (ΔΨm)

Changes in the mitochondrial membrane potential (ΔΨm) were measured in the treated K562 and HeLa cells and nontreated (control) cells. The results show that all investigated compounds **1–4** induced significant disruption of mitochondria (JC-monomers) in both cell lines (Fig. 3; Supplementary material, Fig. S2) compared to control cells.

Leukemia cells (K562) show greater changes in ΔΨm than HeLa cells. In K562 cells observed ΔΨm changes were between 73% (in the cells treated by ligand **1**) to 81.4% (in the cells treated by salt **3**). Similar percentages of JC-1 monomers were present in HeLa cells exposed to salt **3** (65.77%) and in the cells exposed to the mixture **4** (66.88%) and in K562 cells (81.44% *vs.* 80.87%). Ligand **1** caused least ΔΨm changes, in HeLa cells for 54.43% cells and 73.2% in K562 cells, compared to other compounds.

4. Discussion and conclusions

Development of metal complexes, especially non-platinum complexes, as new chemotherapeutics or diagnostic agents has rapidly increased in recent times [35]. Complexes containing biologically important copper ions are promising agents in the cure of tumor diseases and copper ions are also known as very important for many processes in the high proliferative cells such are tumor cells [36,37].

In an attempt toward the development of metal-based anticancer drug the copper(II) complex of novel pyrimidine nucleobase derivative containing a sulfonamide pharmacophore ($Cu(1\text{-}TsC\text{-}N3)_2Cl_2$, complex **2**) was tested on antiproliferative capacity on carcinoma and leukemia cell lines and obtained results were compared with cytotoxic effects of free ligand **1**, $CuCl_2$ x $2H_2O$ salt (salt **3**) and the mixture **4** (salt **3** and ligand **1** in a ratio 1:2). We found that molecular complex **2** significantly improved inhibition of tumor cells' growth in comparison with effect of free ligand **1**. Further, leukemia K562 cells were more sensitive to complex **2** compared to lymphoma and carcinoma cell lines (Table 1) though almost the same cytotoxic effects displayed the salt **3**. To shed some light into mechanisms of observed cytotoxicity we measured intracellular copper concentration in cells treated with complex **2**, salt **3** and the mixture **4**. Based on the cytotoxicity results (Table 1) the K562 and HeLa cells were chosen and treated by tested compounds. We found that the intracellular copper concentration significantly elevated in by complex **2** treated K562 cells after 2 h of incubation by 7.83 times compared to control cells, by 2 times compared to the cells treated with salt **3**, and by 4 times compared to the cells treated by the mixture **4**. It should be emphasized that statistically significant difference between copper concentration in K562 and HeLa cells (7.83 times vs 5.4 times) treated by complex **2** was observed. The intracellular copper concentration also raised in Hela cells treated by all tested compunds **1–4**. In HeLa cells treated with salt **3** and with the mixture **4** copper concentrations increased as those in treated K562 cells. The obtained results could be explained by the fact that copper enters into cells in a form of molecular complex **2** by independent system which enables biologically active copper compounds to penetrate the cell surface without binding to other agents opposite to coordination compounds of other metals [36]. This mechanism is different from the mechanism of uptake of extracellular copper(II) ions [38,39], where the copper(II) is reduced to copper(I) by metalo-reductases located on the cell's surface before entering the cell and susequently transported into the cell mainly by specific copper transporter. The significantly lower growth inhibition of the normal cells caused by salt **3** compared to its effect on the tumor cells found in this study is in accord with earlier observations [40,41].

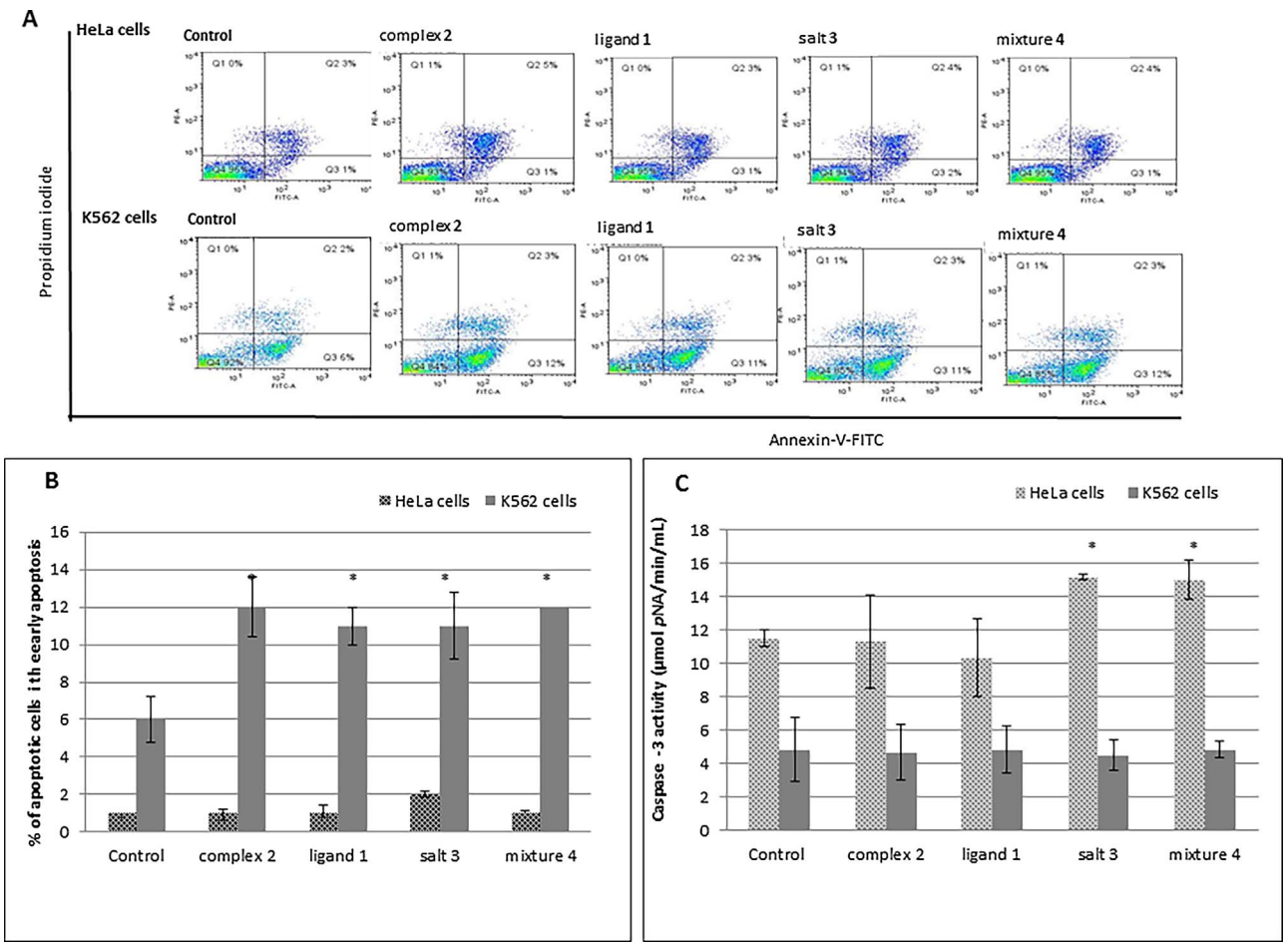


Fig. 2. Induction of apoptosis in HeLa and K562 cells. Cells were cultured 24 h in the presence of 1×10^{-5} M complex **2**, free ligand **1**, salt **3** and the mixture **4**. respectively. Control cells were nontretaed cells. After dying by annexin V-FITC and propidium iodide cells were analyzed using flow cytometry. (A) Dot plot of HeLa and K562 cells flow cytometry analysis with Annexin V-FITC *versus* propidium iodide. The divisions of the plots distinguish late apoptotic/necrotic cells (Annexin V-/PI+, Q2 quadrant), from early apoptotic cells (Annexin V+/PI, Q3 quadrant) and late apoptotic cells (Annexin V+/PI+, Q2 quadrant). The plots in the figure are representative of three independent experiments. (B) Percentage of cells in early apoptosis after treatment and in control nontreated cells. (C) Caspase-3 activity, expressed as µmol of released p-nitroaniline (pNA) per min per ml of cells' lysate. Data represents mean values ± standard deviation (SD) of three independent experiments. *Statistically significant, $p < 0.05$.

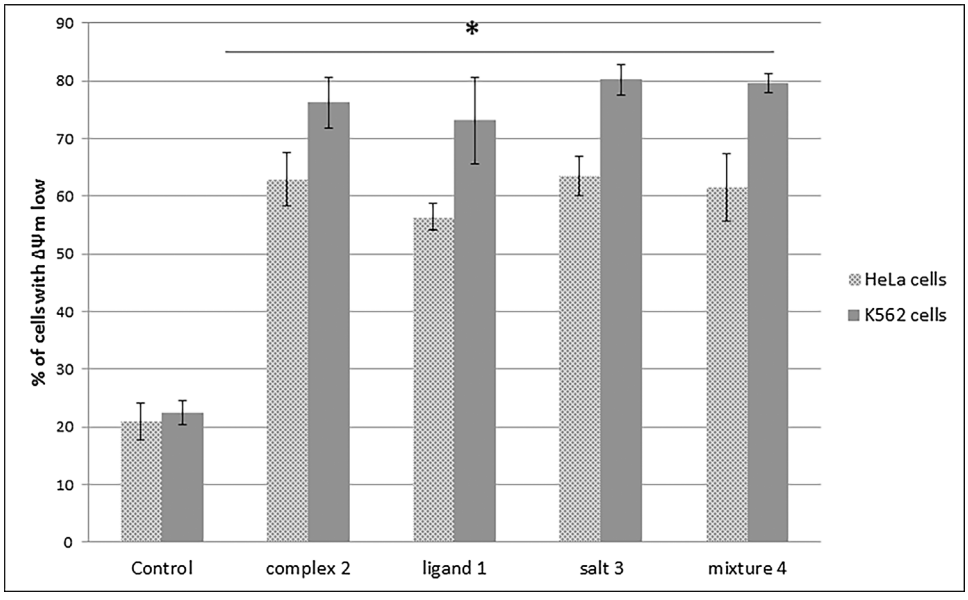


Fig. 3. Flow cytometry detection of changes in the mitochondrial membrane potential (ΔΨm) in K562 and HeLa cells. Cells were treated with free ligand **1,** complex **2**, salt **3**, and the mixture **4** for 24 h respectively. Data represents mean values ± standard deviation (SD) of three independent experiments. *Statistically significant, $p < 0.05$.

Numbers of studies found that copper in the form of organic complexes exhibits cytotoxic activity through cell apoptosis or enzyme inhibition [20,37,42–45,47]. In our previous study a capability of ligand **1** to induce apoptotic cell death was detected [25]. Our current results show that exposure of leukemia cells to tested compounds, during 24 h leads to induction of apoptosis manifested by phosphatidylserine exposure at the outer membrane surface of a cell as a universal process occurring during early apoptosis. Although treated HeLa cells displayed some features of early apoptotic death, more propidium iodide entered into cells (Fig. 2A and B) that could be a sign of a late apoptosis or necrosis.

Apoptosis can be activated by two main signaling caspase dependent pathways: the extrinsic or cytoplasmic pathway, in which cell death receptors are an initiation point of the apoptotic process, and the intrinsic or mitochondrial pathway, where several stimuli act directly on an intracellular target and the mitochondria has a major role [45]. Equally, apoptosis may also be triggered through caspase independent mechanisms, upon different stimuli [48]. Results of this study, presented in Fig. 2C, show that caspase-3 was not activated in the treated K562 cells suggesting that in these cells apoptosis is triggered through caspase independent mechanisms.

Since apoptosis is frequently accompanied by complex mitochondrial changes, alterations in the mitochondrial membrane potential may be an early event in the apoptotic process or, on the contrary, may be a consequence of the apoptotic signaling pathway [46]. In response to multiple distinct intracellular stress conditions, mitochondrial membranes can become permeabilized because of the pore-forming activity of proapoptotic members of the Bcl-2 protein family [47]. Alternatively, mitochondria can lose their structural integrity after the mitochondrial permeability transition, a phenomenon that is initiated at the mitochondrial inner membrane. In both cases, permeabilized mitochondria allow for the release of propapototic proteins into the cellular cytoplasm.

To monitor possible changes in the mitochondrial transmembrane potential ($\Delta\Psi m$), mitochondria were stained with the J aggregate-forming lipophilic cation JC-1, which as a monomer emits green fluorescence and in a reaction driven by the $\Delta\Psi m$ turns into a red fluorescence-emitting dimer, thereby allowing the simultaneous analysis of the total mitochondrial mass per cell (green fluorescence) and of $\Delta\Psi m$ (the quotient of red/green fluorescence). In our study, treatment of both, K562 and HeLa cells with 1×10^{-5} M compounds **1–4** during 24 h increased of JC-1 red/green fluorescence that documents the significant breakdown of the mitochondrial membrane potential ($\Delta\Psi m$). Given results suggest that disruption of mitochondrial membrane potential produced by complex **2** and other tested compounds can lead to cytotoxicity and cell death by apoptosis and/or necrosis as we shown in Fig. 2. These observations can be explained by the fact that exposure to excessive copper ions leads to necrosis or apoptosis through increased generation of reactive oxygen species (ROS) production, oxidation of lipids, or proteins *via* Fenton or Haber-Weiss reactions [38].

Obtained results lead to the conclusion that copper ions have a significant role in apoptosis which is reflected in the increased antiproliferative activity of the complex **2** in comparison to the free ligand **1.** They also indicate that induced apoptosis could be promoted by mitochondria mediated way, but this step should be investigated in more detail. The results of this study contribute to better understanding of interaction of metal ions and cytosine derivatives and could be of importance in future metallodrug design.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

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