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Gold(III) complexes with esters of cyclohexyl-functionalized ethylenediamine-*N*,*N*′-diacetate

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ABSTRACT

Six novel gold(III) complexes containing *O*,*O*′-dialkyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate ([$AuCl_2$\{(*S*,*S*)-R_2eddch\}]PF_6, R = Me, Et, *n*-Pr, *n*-Bu, *i*-Bu, *i*-Am; **1**–**6**, respectively) were synthesized and characterized by elemental analysis, UV/Visible, IR and NMR spectroscopy, mass spectrometry and differential pulse voltammetry. Density functional theory (DFT) calculations confirmed that diastereoisomer with the *N*,*N*′ atoms configured (*S*,*S*) was the most stable. *In vitro* antiproliferative activity was determined against human cervix adenocarcinoma HeLa and human myelogenous leukemia K562 tumor cell lines, as well as against rested and stimulated normal immunocompetent human peripheral blood mononuclear cells (PBMC) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Complex **6** expressed the highest activity against K562 cells (IC_{50} = 3.8 ± 0.5 μM). Apoptosis, seen as condensation of HeLa cell nuclei was the mode of cell death induced by complexes **2**–**6**. Complexes **3**–**6** induced death of K562 cells inhibiting cell entry in mitosis.

1. Introduction

During the last 4 decades, cisplatin, one of the most successful chemotherapeutics has been used for the treatment of numerous types of cancers (ovarian, testicular, head–neck and bladder tumors) [1–6]. However, its clinical usefulness has frequently been limited by severe side effects, such as nephro-, oto- and neurotoxicity, as well as by the development of resistance. These drawbacks have provided the motivation for alternative chemotherapeutic strategies [7–11].

Upon intake, cisplatin is transported through blood stream and excess of chloride ions (~100 mM) prevents most of ligand substitution reactions. Nevertheless, some ligand substitution reactions occur and they are held responsible for acute toxicity [7]. In the cell, concentration of chloride ions is much lower and allows substitution of chlorido ligands by water and evolved hydrolytic species are considered as active cisplatin species [4,12,13].

Activity of cisplatin is based on the ability of platinum to bind to DNA, thus preventing replication and leading to cell death [6]. Earlier studies showed that active hydrolytic cisplatin species have a high affinity for guanosine (G), mostly forming pGpG and pApG intrastrand cross-links adducts [14]. In addition, cisplatin might react with glutathione and other sulphur-containing peptides, which deactivate this chemotherapeutic within tumor cells [3].

The success of metallodrugs is closely linked with the proper choice of ligands, as they play a crucial role in modifying reactivity and lipophilicity, in stabilizing oxidation states and in imparting substitution inertness [15].

R_2edda-type ligands represent the group of *O*,*O*′-dialkyl esters of ethylenediamine-*N*,*N*′-diacetic/dipropanoic (dicyclohexylpropanoic)/dibutanoic/dipentanoic acids. As expected, coordination to a metal ion is κ^2*N*,*N*′ (Fig. 1) [16–23]. High *in vitro* antitumor activity by two platinum(IV) complexes [$PtCl_4$(*n*-Bu_2eddp)] and [$PtCl_4$(*n*-Pe_2eddp)] (Fig. 1, b) was demonstrated against L929 fibrosarcoma and U251 astrocytoma tumor cells [16]. Complexes [$PtCl_4$(R_2edda)] (Fig. 1, a) expressed a moderate antitumor action on a cisplatin-resistant cell line 1411HP [17]. Platinum(IV) complexes were found to be more active than corresponding platinum(II) complexes, with the exception of platinum complexes with (*S*,*S*)-R_2eddl ligands against CLL cells (Fig. 1, d), thus having higher activities than cisplatin [20]. However, platinum(IV) complexes of (*S*,*S*)-R_2eddip were generally more active against HeLa, Fem-x and K562 cells than those of (*S*,*S*)-R_2eddl ligands, but 2–5 times less active in comparison with cisplatin (Fig. 1, c) [21].

Recently, antitumor action of two analogues of these highly active platinum(IV) complexes with shorter alkyl chain in ester function, [$PtCl_4$(R_2eddp)] (R = Et and *n*-Pr) (Fig. 1, b) was investigated. The

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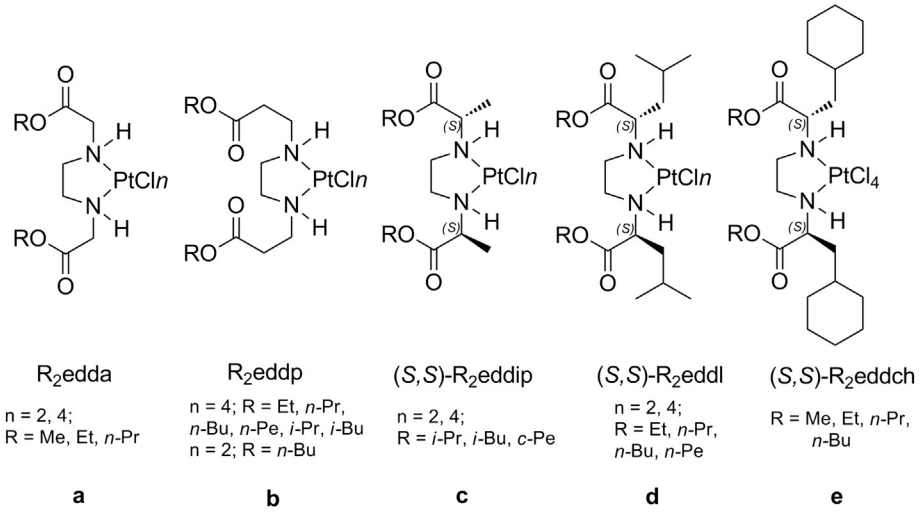


Fig. 1. Complexes of platinum(IV) and platinum(II) with R_2edda-type ligands.

results revealed that platinum(IV) complexes may act as drugs by interacting with DNA, or as prodrugs that could be activated *in vivo* within the cell by reduction to corresponding reactive platinum(II) species [19].

Lately, our investigations were mainly concentrated on the preparation, characterization and antitumor activity of platinum(IV) compounds with cyclohexyl-functionalized R_2edda-type ligands [24].

Among non-platinum compounds for cancer treatment, complexes containing palladium, ruthenium, gold and silver, even titanium and tin compounds have been reported [25–34].

Gold(III) complexes have a long tradition in applications in medicine as drugs [35–39]. During the early 90s, there was a revival of interest toward gold(III) complexes with improved stability and encouraging pharmacological properties.

Platinum(II) and gold(III) are isoelectronic and isostructural (d^8 system, square-planar geometry), thus gold compounds could show a model of binding to DNA similar to cisplatin [40,41]. However, gold(III) complexes were found to have different properties in comparison with the platinum(II) compounds [42–45], because of their poorer solubility and higher kinetic lability than corresponding platinum(II) complexes. Furthermore, although they are easily reducible to metallic gold, some of the gold(III) compounds expressed a high cytotoxicity [46].

Antitumor activity of various gold(III) complexes containing ethylenediamine derivatives has also been recently reported [38,47,48]. The mechanisms of action of gold(III) complexes seem to be innovative and substantially different from those of cisplatin. They have a weak binding affinity for DNA which is the primary target for platinum(II) antitumor drugs. Also, gold(III) complexes have shown high reactivity toward different model proteins [49,50]. In fact a seleno-enzyme, thioredoxin reductase (TrxR) playing a key role in the regulation of the overall intracellular redox equilibrium is a very specific target for gold(III) complexes [45,51]. It is important to note that gold(I) species produced by gold(III) reduction *in vivo* could represent active metabolites [40].

In the present study, synthesis, characterization and cytotoxic activity of six novel gold(III) complexes with cyclohexyl functionalized R_2edda-type ligands are presented. To elucidate the features determining the preferred configuration of R_2edda-type ligands coordinated to the gold(III) complexes, density functional theory (DFT) analyses were performed. Additionally, *in vitro* antitumor activity of novel complexes **1**–**6** against human adenocarcinoma HeLa, human myelogenous leukemia K562 tumor cell lines and rested and stimulated normal immunocompetent cells (PBMC) was investigated. The mode of HeLa cell death induced by **2**–**6** was also studied, as well as cell cycle distribution of K562 cells upon treatment with these complexes.

2. Experimental

2.1. Chemistry

2.1.1. Starting agents

The methyl, ethyl, *n*-propyl, *n*-butyl, isobutyl and isoamyl esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid were synthesized according to described method [24]. Na[$AuCl_4$] was synthesized by the standard procedure [52].

2.1.2. Measurements

Elemental analyses were performed on an Elemental Vario EL III microanalyzer. A Nicolet 6700 FT-IR spectrometer and ATR technique were used for recording infrared spectra. NMR spectra were recorded on Varian Gemini 200 or Bruker Avance III 500 spectrometers. Chemical shifts for ^{1}H and ^{13}C NMR spectra were referenced to residual ^{1}H and ^{13}C present in $CDCl_3$. Mass spectra were recorded with a 6210 Time-of-Flight LC–MS instrument (G1969A, Agilent Technologies) in CH_3CN. A GBC UV/Vis Cintra 6 spectrometer was used for electronic spectra of complexes dissolved in $CHCl_3$ at 1×10^{-4} M. Reagents and solvents were of commercial reagent grade quality and used without further purification. Electrochemical studies were performed by differential pulse voltammetry using CHI-760B instrument with a scan rate of 20 mV/s at room temperature. The working electrode was a platinum electrode (Model 6.1204.120), the reference electrode was non-aqueous Ag/AgCl electrode (Model CHI 112) and the counter electrode was a platinum wire (model CHI 115). The complexes, **1**–**6** were dissolved in DMSO in concentrations of 1.0 mM containing $LiClO_4$ as supporting electrolyte.

2.2. Synthesis of complexes [$AuCl_2${(*S*,*S*)-R_2eddch}]PF_6, **1**–**6**

Suspensions containing 0.126 mmol of each ligand, [(*S*,*S*)-H_2 R_2eddch}]Cl_2, R = Me (0.059 g), or Et (0.063 g), or *n*-Pr (0.067 g), or *n*-Bu (0.070 g), or *i*-Bu (0.070 g), or *i*-Am (0.073 g), **L1**·2HCl–**L6**·2HCl, respectively, were prepared in methanol (4 ml) and $LiOH \cdot H_2O$ (0.011 g, 0.252 mmol) was added. After stirring for 1 h in the dark, at ambient temperature, deprotonated ligands dissolved completely. Then, Na[$AuCl_4$]·$2H_2O$ (0.050 g, 0.126 mmol) solution in methanol (4 ml) was introduced and followed by adding solid NH_4PF_6 (0.062 g, 0.378 mmol). The resulting red solution was evaporated *in vacuo* and the yellow product washed with excess of water.

Experimental data of the complexes **1**–**6** are included in Supplementary materials.

2.3. Computational details

Geometry optimizations were performed with the Gaussian 03 package [53]. All structures were optimized using the MPW1PW91 functional [54]. The Stuttgart/Dresden (SDD) basis set for all atoms was employed in the calculations [55,56]. All systems were optimized without symmetry restrictions. The resulting geometries were characterized as equilibrium structures by the analysis of the force constants of normal vibrations. Supplementary data associated with the quantum chemical calculations can be obtained from the authors upon request.

2.4. In vitro studies

2.4.1. Cell culture

Human cervix adenocarcinoma HeLa cells were cultured as monolayers in the nutrient medium, while human myelogenous leukemia K562 cells were maintained as suspension culture. The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

PBMC were separated from the whole heparinized blood of healthy volunteers by Lymphoprep™ gradient centrifugation. Interfaced cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g/l gelatin polymers, pH 7.4) were counted and resuspended in nutrient medium.

2.4.2. Cell sensitivity analysis

HeLa cells were seeded (2000 cells per well) into 96-well microtiter plates and 20 h later, after the cell adherence, five different concentrations of investigated compounds were added to the wells. For **1** and **2**, final concentrations were in the range from 50 to 3.12 μM, and for **3**, **4**, **5**, and **6** they were in the range from 25 to 1.56 μM. Only nutrient medium was added to the cells in the control wells. Investigated compounds were added to suspension of leukemia K562 cells (5000 cells per well) 2 h after the seeding, in the same final concentrations applied to HeLa cells. All experiments were done in triplicate. Nutrient medium with corresponding concentrations of compounds, but void of cells, was used as blank.

PBMC were seeded (150,000 cells per well) into nutrient medium or in nutrient medium enriched with (5 μg/ml) phytohemaglutinin, PHA (INEP, Belgrade, Serbia) in 96-well microtiter plates and 2 h later, investigated compounds were added to the wells, in triplicates, to five final concentrations, except the control wells where a nutrient medium only was added to the cells. Nutrient medium with corresponding concentrations of compounds, but void of cells, was used as blank.

Inhibition of growth was determined by MTT test 72 h after the drug addition [57,58]. Briefly, 10 μl of MTT solution (5 mg/ml in PBS — phosphate-buffered saline) was added to each well. Samples were incubated for further 4 h at 37 °C in 5% CO_2 and humidified atmosphere. Then, 100 μl of 10% SDS was added to the wells. Absorbance (A) at 570 nm was measured the next day. To get cell survival (%), A of a sample with cells grown in the presence of various concentrations of investigated agent was divided with control absorbance, A_c, (the A of control cells grown only in nutrient medium) × 100. It was implied that A of blank was always subtracted from A of a corresponding sample with target cells. Concentration IC_{50} was determined as the concentration of a drug that inhibited cell survival by 50%, compared with vehicle-treated control.

2.4.3. Preparing cells for fluorescence imaging

Morphological features of HeLa cell death induced by investigated compounds were analyzed after staining of the treated cells with mixture of acridine orange (AO) and ethidium bromide (EtB) [59]. Briefly, 1×10^5 of HeLa cells was seeded on glass slide in Petri dishes, and treated on the next day with 2 × IC_{50} concentration of investigated compounds for 24 h. Then, the cells were stained with 15 μL of a mixture of working concentration of AO/EB (3 μg/ml AO and 10 μg/ml EB in PBS) and visualized under a fluorescence microscope using a fluorescein isothiocyanate (FITC) filter set.

2.4.4. Cell cycle determination

Target K562 cells were incubated in the presence of two different concentrations of investigated compounds (IC_{50} and 2 × IC_{50}) for 24 and 48 h. After appropriate incubation time, the target cells were collected, washed and fixed in 70% ethanol on ice. Samples were stored at −20 °C for at least one week before staining. K562 cells were washed in PBS, then resuspended in PBS containing RNAse A (final concentration 100 μg/ml), and incubated at 37 °C for 30 min. Then propidium iodide (PI) (final concentration 40 μg/ml) was added and samples were incubated at 37 °C for 30 min. Cell cycle phase distribution was determined using a FACSCalibur Flow Cytometer (BD Biosciences Franklin Lakes, NJ, USA). Acquired data (10.000 events collected for each sample) were analyzed using CELLQuest Software (BD Biosciences).

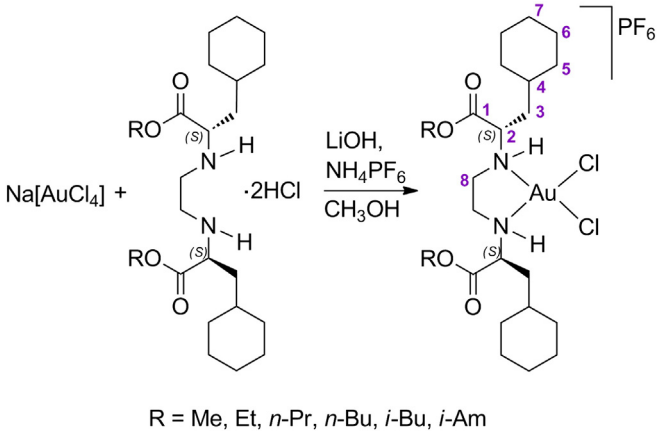
3. Results and discussion

3.1. Synthesis and characterization

In the reaction of $Na[AuCl_4]\cdot 2H_2O$ and an equimolar amount of corresponding ligand (Scheme 1) in methanol, desired complexes **1**–**6**, were obtained after addition of ammonium hexafluorophosphate to the reaction mixture. The resulting yellow product was washed with excess of water. The prepared complexes were soluble in methanol, ethanol, acetone, dichloromethane, chloroform, dimethyl sulfoxide and acetonitrile.

IR spectra showed strong ν(C═O) absorption bands stretching from 1729 to 1734 cm^{-1} (**1**–**6**) [21,60], similar to the corresponding free ligands ([24], J.M. Poljarević et al., manuscript in preparation), thus indicating that the coordination of the carboxylic oxygen to the metal center can be excluded. Asymmetric $\nu(-CH_3/-CH_2)$ stretching vibrations of moderate intensities were recorded within the range from 2851 to 2930 cm^{-1}. In addition, the change of asymmetric C – N vibration values from 803–823 cm^{-1} (free ligands) to 847–850 cm^{-1} (novel complexes) indicates that ligands coordinate *via* nitrogen atoms [60]. The data obtained using UV/Vis spectroscopy showed absorptions around 320 nm ($\varepsilon = 31{,}250\ cm^{-1}$) which could be assigned to Cl → Au charge transfer [61]. According to the crystal field theory of d^8 complexes the lowest unoccupied molecular orbital (LUMO) is $d(x^2 - y^2)$, so ligand to metal charge transfer could be due to $p_\sigma \rightarrow d(x^2 - y^2)$ transition.

^1H and ^{13}C NMR spectra were analogous to those of previously characterized platinum complexes with R_2edda-type ligands [17–19,24]. Selected NMR data is listed in Table 1. Chemical shifts of cyclohexyl hydrogen atoms (C^4H, $C^{5-7}H_2$) were observed between 0.9 and 1.9 ppm. The hydrogen atoms belonging to the secondary amino groups



R = Me, Et, *n*-Pr, *n*-Bu, *i*-Bu, *i*-Am

Scheme 1. Synthesis of complexes **1**–**6**.

Table 1
Selected ^{1}H and ^{13}C NMR data (δ in ppm) for complexes, **1–6**.

Complexes	^{1}H			^{13}C			
	$C^{8}H_2$	CH_2O/CH_3O	$C^{2}H$	$C^{8}H_2$	$C^{2}H$	$C^{1}OO$	CH_2O/CH_3O
1	3.57	3.89	4.06	44.3	59.2	170.6	54.0
2	3.55	4.34	4.02	44.1	59.2	170.1	63.5
3	3.48	4.23	3.98	44.4	59.2	170.7	68.7
4	3.57	4.30	4.04	44.2	59.3	170.3	67.3
5	3.53	4.06	4.06	44.3	59.3	170.5	73.3
6	3.43	4.30	3.92	44.5	59.2	171.0	65.8

of all complexes appeared in the ^{1}H NMR spectra at approximately 4.7 ppm. The resonances of ethylene hydrogen atoms ($C^{8}H_2$) showed coordination induced shifts (*ca.* 0.5 ppm), which also indicates that the coordination occurred *via* nitrogen atoms. In ^{13}C NMR spectra chemical shifts arising from ester carbon atoms were found at the expected positions for this class of compounds. Chemical shift around 170 ppm (C^{1}) clearly demonstrates that oxygen atoms are not participating in coordination. Resonances of carbon atoms from ethylenediamine moiety (C^{8}) of the complexes were shifted upfield in relation to those of the free ligand. Mass spectra of all complexes (positive mode) revealed the presence of $[M]^{+}$ ion.

3.2. Quantum chemical calculations

DFT calculations were conducted for the isomers arising from $\kappa^{2}N,N'$ coordination of (*S*,*S*)-R_2eddch (R = Me, Et, *n*-Pr, *n*-Bu, *i*-Bu, *i*-Am) to $AuCl_2$ fragment. Equilibrium structures are shown in Fig. 2. All structures were fully optimized without any symmetry constraints. The calculated results for all complexes showed that the (*S*,*S*)-**1c**–(*S*,*S*)-**6c** diastereoisomers appear to be structurally and synthetically feasible. Differences in energy between the most stable (*S*,*S*) isomer and (*R*,*R*) and (*R*,*S*) isomers ranged between 4.2–5.4 and 2.9–5.4 kcal/mol, respectively, and formation of these isomers should not be expected. The results obtained by NMR spectroscopy (within the sensitivity limits of this method) revealed the presence of a single isomer and DFT calculations indicated that it could be assigned as (*S*,*S*).

3.3. Electrochemistry

Redox potentials of complexes **1–6** determined by electrochemical studies (*vs.* Ag/AgCl) ranged from −237 to −304 mV. The gold(III) to gold(I) reduction represents an irreversible process followed by the loss of the chlorido ligands [62,63]. Reduction occurs more readily in investigated complexes containing longer alkyl substituent (R = *n*-Bu, *i*-Bu, *i*-Am). The occurrence of the Au^{III}/Au^{0} reduction can be rejected due to the lack of gold metal at platinum electrode. Overall, strong correlation between reduction potentials and biological activity was not detected.

3.4. In vitro cytotoxicity

The representative graphs showing action of various concentrations of investigated complexes on HeLa and K562 cell survival, determined by MTT test, upon 72 h of continuous agent action are shown in Fig. 3. The examined agents expressed an antiproliferative effect against investigated target cancer cell lines, as well as on normal human stimulated and non-stimulated PBMC, in a dose-dependent manner.

The cytotoxic action of all investigated gold(III) complexes (Table 2), was the most pronounced against myelogenous leukemia K562 cells. The intensity of this action decreased when HeLa, and stimulated or resting PBMC were used as target cells.

The replacement of methyl by ethyl group in ester moiety (**1** → **2**) led to increased cytotoxic activity of investigated complexes against both malignant and non-malignant cells. The substitution of ethyl with *n*-propyl group (**2** → **3**) in gold(III) complexes didn't affect significantly antiproliferative action against all examined cell lines. The

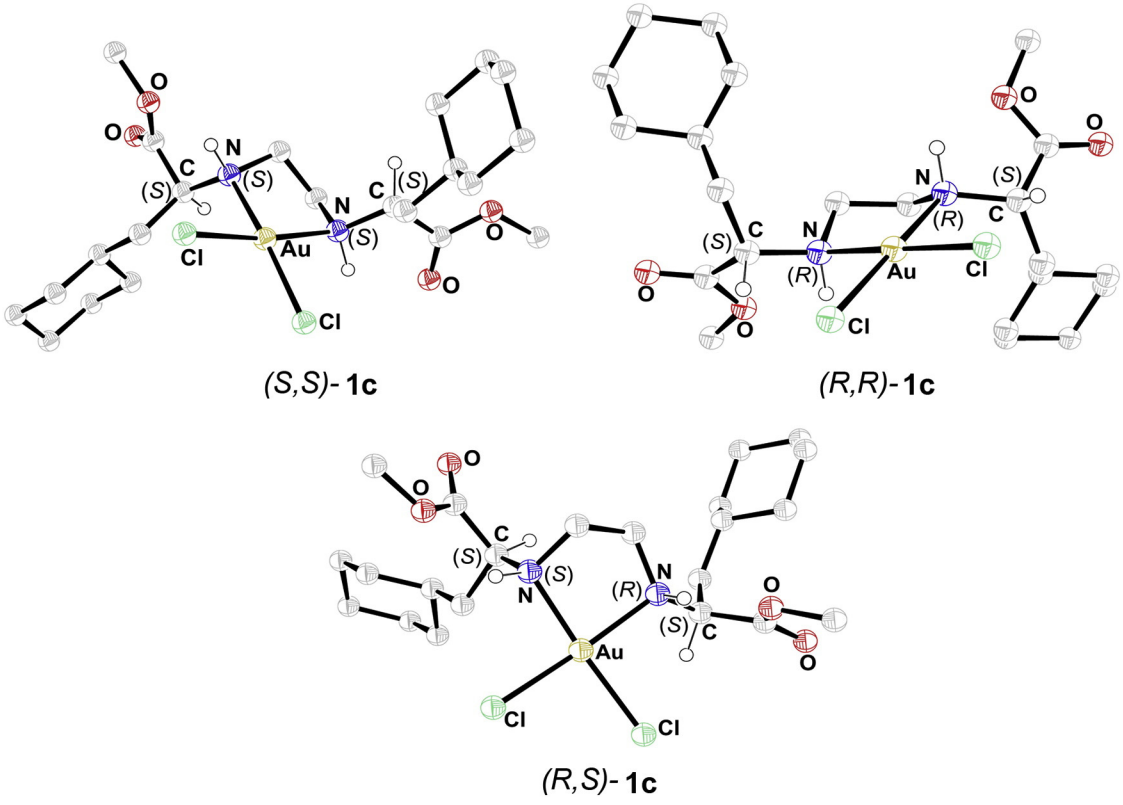


Fig. 2. Calculated structures of **1c**–**6c**. H atoms, except those bonded to chiral atoms, are omitted for clarity.

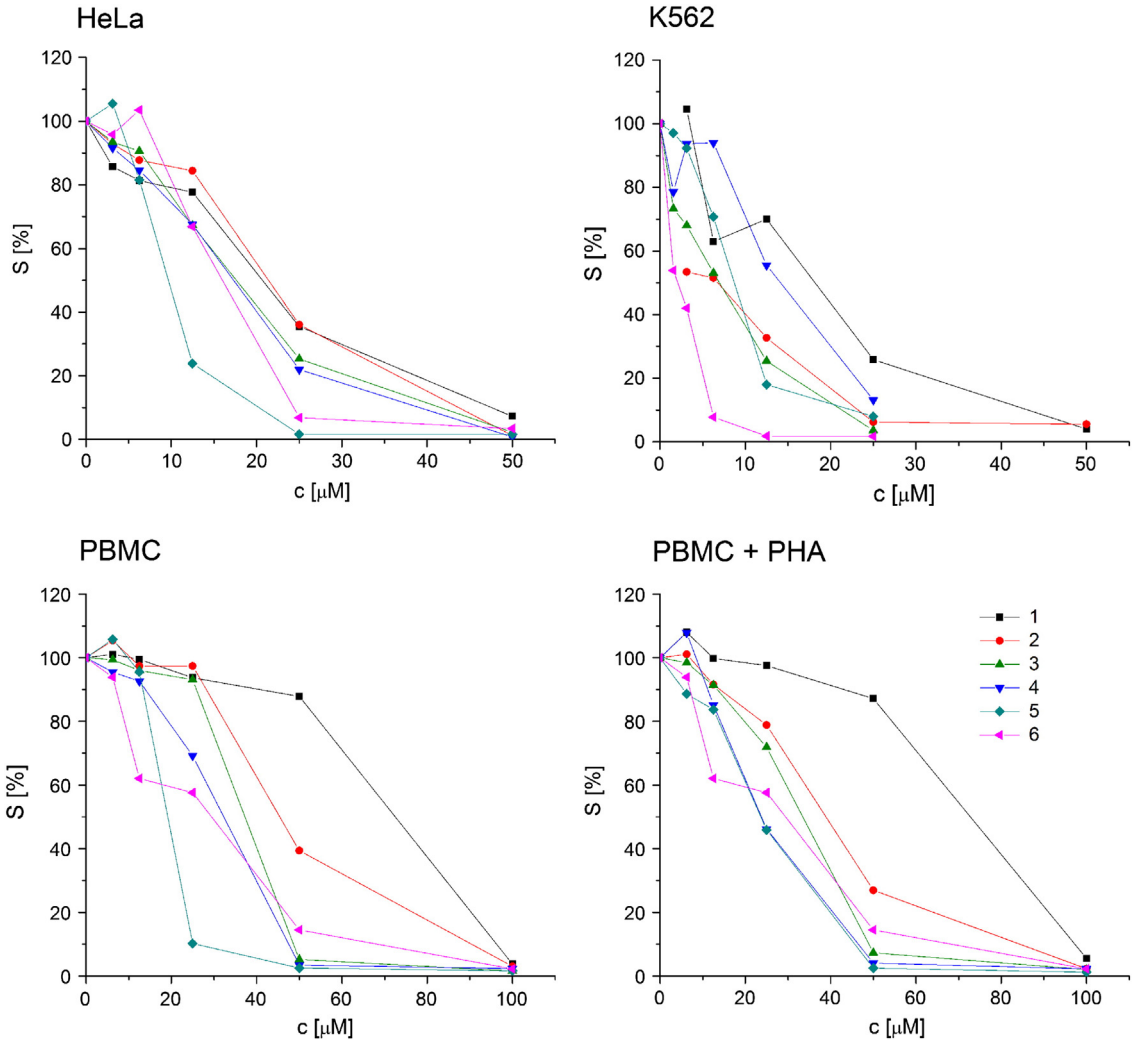


Fig. 3. Representative graphs show survival of HeLa, K562, PBMC, and PBMC + PHA cells grown for 72 h in the presence of increasing concentrations of investigated compounds, determined by MTT test.

substitution of *n*-propyl with *n*-butyl or isobutyl group (**3** → **4**, **5**) led to similar cytotoxic action. Complexes **2**, **4** and **5** expressed the highest activity against HeLa cell line comparable to that of [$PtCl_4$(*n*-Bu_2eddp)] [18]. On the other hand, the same complexes exhibited a stronger antiproliferative effect against K562 cell line as compared to HeLa cell line. Furthermore, *in vitro* activity of **2**, **3** and **5** was comparable to that of [$PtCl_4$(*n*-Bu_2eddp)] complex. Interestingly, complex **6** was quite inactive against HeLa, but was found to be the most effective against K562 cell line. The activity of **6** was higher in relation to the reference compound cisplatin and [$PtCl_4$(*n*-Bu_2eddp)] [18].

Stimulated PBMC were found moderately sensitive to toxic action of **6**, while resting PBMC were less sensitive. Analogously, a similar trend was observed with all examined compounds on rested and stimulated PBMC, implying selectivity in their action (Table 3). Highest selectivity of complexes **1**–**6** against malignant than normal cells was observed when K562 tumor cells were used. Compound **6** expressed the greatest activity against K562 cell line. Complexes **1** and **2**, with shorter ester chains (R = Me, Et), were also found to be considerably selective toward K562 cells.

Table 2
IC_{50} values (μM)[a] of the investigated gold(III) complexes **1**–**6**, [$PtCl_4$(*n*-Bu_2eddp)] [18] and cisplatin on HeLa and K562 cells, and on PBMC and PBMC stimulated with PHA determined by MTT test after 72 h of treatment.

Complex	IC_{50} [μM]			
	HeLa	K562	PBMC	PBMC + PHA
1	29.03 ± 4.22	15.03 ± 4.44	76.07 ± 5.16	61.40 ± 10.76
2	16.76 ± 4.31	8.35 ± 2.27	42.47 ± 4.18	24.39 ± 3.46
3	20.34 ± 4.02	7.77 ± 2.04	37.77 ± 2.26	31.04 ± 2.18
4	16.97 ± 1.09	12.05 ± 1.92	23.42 ± 1.96	21.30 ± 3.49
5	17.74 ± 2.79	10.73 ± 2.07	31.00 ± 8.78	21.94 ± 2.73
6	26.35 ± 3.88	3.76 ± 0.55	29.35 ± 13.17	21.31 ± 9.34
[$PtCl_4$(*n*-Bu_2eddp)]	14.88 ± 2.36	7.08 ± 2.00	14.49 ± 5.84	10.56 ± 4.45
Cisplatin	4.47 ± 0.30	5.77 ± 0.35	33.60	26.51 ± 5.74

[a] Mean values ± SD (standard deviation) from three experiments.

3.5. Morphological analysis of HeLa cell death

AO/EB double staining assay was applied to determining the mode of cell death induced by gold(III) complexes in HeLa cell line. Selected cell lines were pretreated for 24 h with the examined complexes in concentrations equal to 2 × IC_{50} for each agent separately (Fig. 4).

Moreover, no significant impact on cell morphology was observed when HeLa cells were treated with complex **1**. Microphotographs of treated HeLa cells showed that compounds **2**–**6** induced condensation of nuclei indicating apoptosis as a mode of cell death. Explicitly, in the case of complex **2** there was a sign of early apoptosis seen as cell

Table 3
Selectivity index.

Complex	IC_{50}(PMBC)/IC_{50} (cell line)		IC_{50}(PMBC + PHA)/IC_{50} (cell line)	
	Cell line			
	HeLa	K562	HeLa	K562
1	2.62 ± 0.42	5.06 ± 1.53	2.11 ± 0.48	4.09 ± 1.41
2	2.53 ± 0.70	5.09 ± 0.29	1.46 ± 0.43	2.92 ± 0.89
3	1.86 ± 0.83	4.86 ± 1.31	1.53 ± 0.32	3.99 ± 1.08
4	1.38 ± 0.14	1.94 ± 0.35	1.26 ± 0.22	1.77 ± 0.40
5	1.75 ± 1.83	2.89 ± 0.99	1.24 ± 0.25	2.04 ± 0.47
6	1.11 ± 0.23	7.81 ± 3.68	0.81 ± 0.37	5.67 ± 2.62
[$PtCl_4$(*n*-Bu_2eddp)]	0.97 ± 0.39	2.05 ± 0.96	0.71 ± 0.32	1.49 ± 0.76
Cisplatin	7.52	5.82	5.93 ± 1.35	4.59 ± 1.03

rounding with a beginning of nuclei condensation. Cell membrane blebbing, as a characteristic of early apoptosis stage, can be seen in HeLa cell line treated with **3**. The application of complexes **4**, **5** or **6** induced the condensation of cell nuclei.

3.6. Cell cycle analysis

To further examine the mechanisms of action of novel compounds, determination of cell cycle distribution was performed on K562 cells. As seen from Fig. 5, the number of K562 cells in G1 phase didn't change significantly after 24 and 48 h exposure to gold(III) complexes. Compounds **3**–**6** did not influence significantly (IC_{50} and 2 × IC_{50}) the cells in S phase in either tested time period. Similar effect of the compounds was observed in Sub-G1 fraction after 24 h treatment with IC_{50} concentrations of **3**–**6**. On the other hand, after 24 h exposure of K562 cells to 2 × IC_{50} concentrations, the number of cells in Sub-G1 increased. Higher effect of **3**–**6** on Sub-G1 fraction was noticed after 48 h

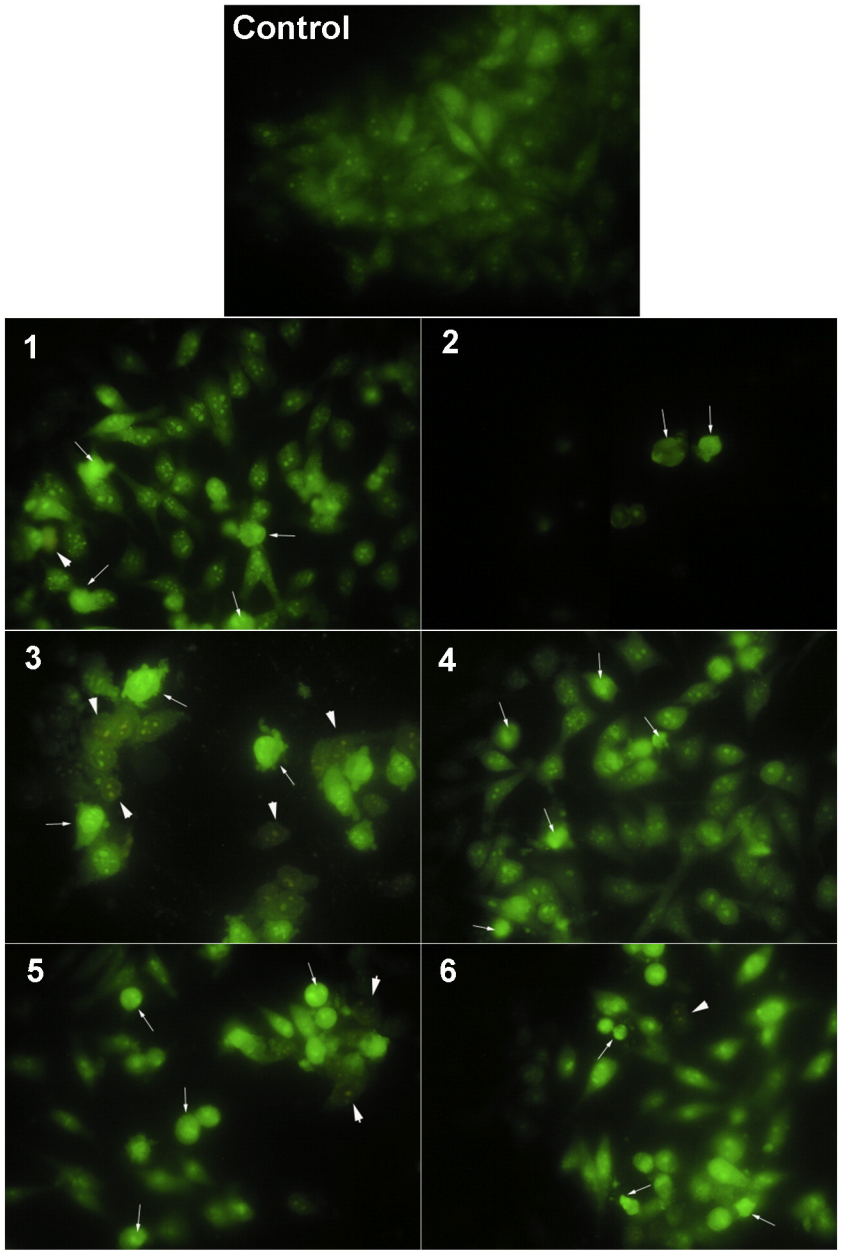


Fig. 4. Induction of apoptosis in HeLa cells by investigated gold(III) complexes: acridine orange and ethidium bromide stained cells. Untreated control cells (control), and cells treated with designated compounds, **1**–**6**. Arrowheads indicate cells with degraded material of nucleus with more pronounced nucleoli, while arrows mark apoptotic cells with condensed chromatin.

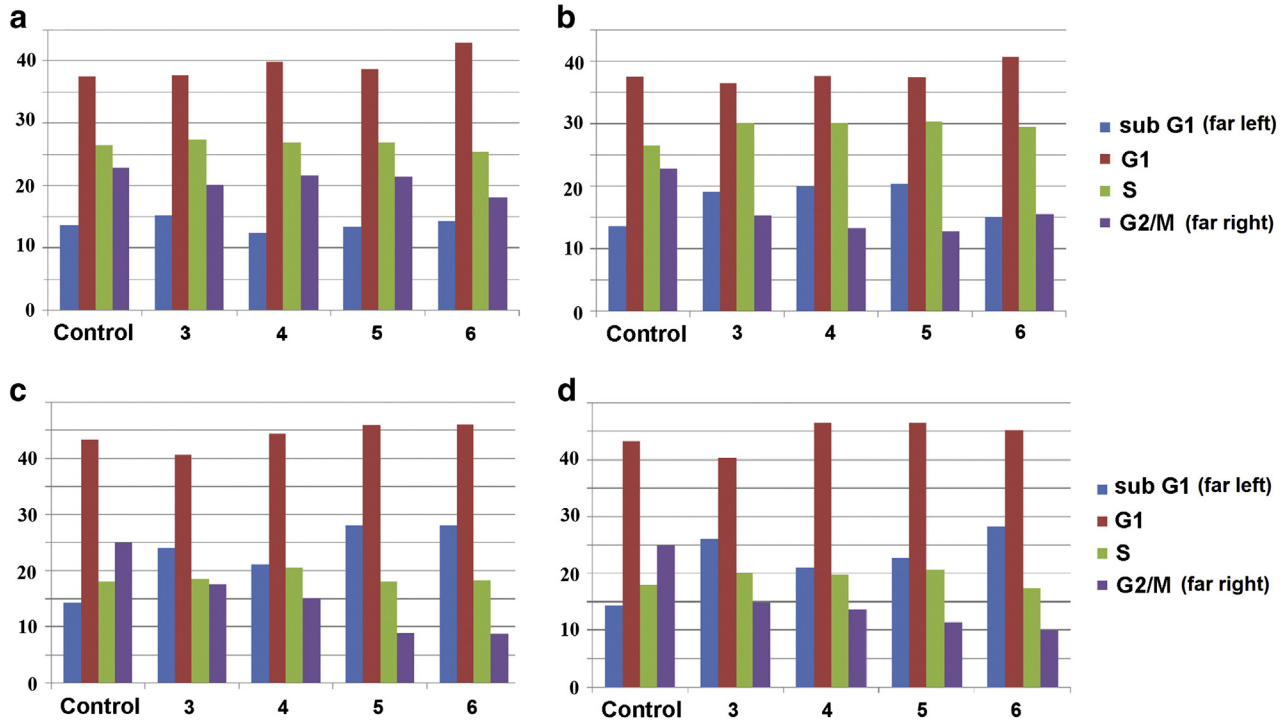


Fig. 5. Cell cycle distribution after 24 (a, b) and 48 h (c, d) continuous action of investigated gold(III) complexes. After exposure to investigated compounds for indicated time periods (concentration corresponded to IC_{50} (a, c) and 2 × IC_{50} (b, d) values).

treatment. Accordingly, reduction of cell population in G2/M phase of cell cycle was observed as compared to the control. These results strongly suggest that the investigated gold(III) complexes acted inhibiting cell entry in mitosis and induced cell death.

4. Conclusions

Syntheses of six novel gold(III) complexes with *O,O′*-dialkyl-(*S,S*)-ethylenediamine-*N,N′*-di-2-(3-cyclohexyl)propanoate ligands, **1–6**, were described. The compounds were characterized by elemental analysis, UV/Vis, IR, NMR spectroscopy, mass spectrometry and differential pulse voltammetry. The IR, and ^{1}H and ^{13}C NMR spectroscopic data suggest ligand chelation *via* nitrogen donor atoms. The gold(III) complexes yielded only a single isomer (*S,S*) and this was confirmed by DFT calculations. Electrochemistry investigations showed that reduction to gold(I) species occurred more rapidly in complexes with a longer alkyl group (R = *n*-Bu, *i*-Bu, *i*-Am).

All investigated compounds were tested for *in vitro* antiproliferative activity against human adenocarcinoma HeLa, human myelogenous leukemia K562 tumor cell lines and normal immunocompetent cells, *i.e.*, human peripheral blood mononuclear cells (PBMC). Among examined complexes, compound **6** expressed the highest activity (IC_{50} = 3.8 ± 0.5 μM) and selectivity index (IC_{50}(PMBC)/IC_{50}(K562) = 7.8 ± 3.7) against K562 tumor cells. Apoptosis, seen as condensation of the nuclei was the mode of HeLa cell death induced by complexes **2–6**. The results obtained by determining the cell cycle distribution of K562 cells suggest that compounds **3–6** inhibited cell entry in mitosis thus inducing cell death. Complex **6** was more active against K562 cells than cisplatin and previously investigated [$PtCl_4$(*n*-Bu_2eddp)].

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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.2013.08.002.

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