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Antileukemic action of novel diamine Pt(II) halogenido complexes: comparison of the representative novel Pt(II) with corresponding Pt(IV) complex

Running title: Antileukemic action of novel diamine Pt(II) complexes

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Abstract

This study presents the synthesis, characterization and antitumor action of five new Pt(II) halogenido, chlorido and iodido, complexes with *edda* type of ligands. *(S,S)*-ethylendiamine-*N*,*N'*-di-2-(3-cyclohexyl)propanoic acid dihydrochloride and its methyl, ethyl and *n*-propyl esters, were prepared according to the previously reported procedure. All investigated complexes were characterized by IR, ESI-MS, (^{1}H, ^{13}C and HMBC) NMR spectroscopy and elemental analysis. Their cytotoxic action was investigated in four human tumor cell lines: promyelocytic (HL-60) and lymphocytic (REH) leukemia, glioma (U251) and lung carcinoma (H460). Cell viability was assessed by acid phosphatase and LDH assay, while oxidative stress and cell death parameters were analyzed by flow cytometry. The results showed that novel Pt(II) complexes exhibited antitumor action superior to precursor ligands, with iodido complexes being more efficient than corresponding chlorido complexes. Human promyelocytic cell line (HL-60) was the most sensitive to antitumor action of all investigated substances, and was used for investigation of the underlying mode of antileukemic action. The investigated Pt(II) complexes showed more potent antileukemic action than corresponding Pt(IV) complex, through induction of oxidative stress and apoptosis, evidenced by caspases (8, 9 and 3) activation and phosphatidylserine externalization.

Keywords: platinum(II) complexes, *edda* ligands, antileukemic, phosphatidylserine, caspases

1. Introduction

Blood cancers (leukemia, lymphoma, myeloma and myelodysplastic syndromes) account for 10.2 % of new malignancies diagnosed in the US in 2016 [1]. Survival rates vary by leukemia subtype, and average 5-year survival is less than 30 % for patients with acute myeloid leukemia. As chemotherapy is used to treat most types of leukemia, the search for new and more effective drugs is ongoing.

The discovery of the potent antitumor activity of cisplatin was the beginning of platinum-based medicinal chemistry [2,3]. Cisplatin binds at the N7 position of guanine and by forming a range of adducts, intrastrand in particular, causes DNA damage and provokes apoptosis [4]. Apoptosis, a type of regulated programmed cell death [5] is a mechanism of action of many chemotherapeutics. Apoptotic cells undergo caspase activation (through extrinsic or intrinsic pathways), phosphatidylserine externalization on the outer leaflet of the

cell membrane, and DNA and cytoplasm fragmentation in the absence of plasma membrane damage. They are quickly engulfed by phagocytic cells in the absence of immune response [6].

Nowadays cisplatin is used in the therapy of lymphomas and myelomas, testicular and ovarian cancer, bladder, melanoma, non-small cell and small cell lung cancer [7]. However, it is not universally effective in all cancer types and causes a number of side effects [8].

In the last four decades scientists have been searching for novel analogues of cisplatin in order to overcome its toxicity and tumor resistance. Initially, new platinum antitumor agents had two *cis* nonleaving amino ligands and two *cis* good leaving groups in their structure. Amino ligands were monodentate or bidentate amines, while the most common leaving groups were chlorido or carboxylato ligands. Only two out of thousands of synthesized and tested platinum compounds, have been introduced into clinical use: oxaliplatin and carboplatin [9,10,11,12] (Figure 1). Carboplatin and oxaliplatin are thought to possess mechanism of action similar to that of cisplatin, which is in essence DNA damage induction. Carboplatin causes fewer side effects compared to cisplatin, while oxaliplatin shows improved performance towards colorectal cancers. Nevertheless, cisplatin still remains the chemotherapeutic agent of choice in a number of malignancies [13,14].

Among numerous complexes investigated so far, diaminedichloridoplatinum(II) complexes are of special importance. A series of asymmetric platinum complexes *cis*-$Pt(LL')Cl_2$ (L = NH_3, L´ = CH_3NH_2, $(CH_3)_2NH$, $C_2H_5NH_2$ and $(C_2H_5)_2NH$ and LL´ = N,N-dimethylethylenediamine) were synthesized and tested in mouse lymphocytic leukemia cells, demonstrating IC_{50} values almost comparable to those of cisplatin [15].

Furthermore, with the aim of increasing lipophilicity, diaminocyclohexane platinum complexes were synthesized and their *in vitro* antitumor activity was widely studied. For example, water-soluble square-planar hydroxylated 1,2-diaminocyclohexane platinum(II) complex possesses the property of inhibiting the growth of tumors in mammals [16]. Another class of diamine platinum(II) compounds with antitumor activity is a group of ethylenediamine-based platinum complexes. In the last decade a number of these complexes were synthesized, using chelating ethylendiamine ligands derived from different amino acids and 1,2-dibromethane [17], including Pt(IV) and Pt(II) complexes with ethylendiamine-*N,N'*-diacetate (*edda*) type ligands. These complexes showed antitumor activity equal to or better than cisplatin on a wide spectrum of tumor cell lines [18,19,20].

The possibility to replace chlorides with iodides within biologically active square-planar platinum(II) complexes has been recently described [21,22]. Namely, Pt−Cl bond more easily

hydrolyzes than corresponding Pt−I bond, contributing to higher reactivity and biological efficacy compared to diiodidodiaminplatinum(II) complexes. The first report on *cis*-$[PtI_2(NH_3)_2]$ documented the lack of antitumor activity in chosen *in vivo* animal models [23]. Nonetheless, several recent studies highlight an unexpected reactivity of iodido Pt(II) and Pt(IV) complexes towards serum albumin and glutathione [24,25,26]. Our recent paper describes the synthesis and antitumor activity of three new platinum(II) iodido complexes with cyclohexyl substituted ethylendiamine type ligands [27]. The report shows that these complexes have a good *in vitro* antitumor activity, comparable with cisplatin. It has also been stated that bulky ligands increase lipophilicity with iodide ligands enabling kinetic stability in physiological conditions, thus leading to better intracellular accumulation and DNA binding, compared to cisplatin [27].

Bearing in mind that cisplatin is still a widely used antitumor agent and that its analogue carboplatin together with other chemotherapeutics is included in phase II trial of relapsed/refractory Hodgkin lymphoma [28], the aim of this report was to synthesize new cisplatin analogues, chlorido-Pt(II) complexes with cyclohexyl substituted ethylendiamine type ligands, compare their activity to analogue iodido-Pt(II) complexes, investigate their antileukemic action and see if there is an agreement with the literature assumption that Pt(IV) complexes are more active compared to analogue Pt(II) complexes.

2. Methods and materials

2.1. Chemistry

2.1.1. Starting substances

(*S*)-2-amino-3-cyclohexyl propanoic acid hydrochloride was purchased from Senn Chemicals (Dielsdorf, Switzerland). *(S,S)*-ethylendiamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid dihydrochloride and its methyl, ethyl and *n*-propyl esters were prepared according to the previously described procedure [20]. $K_2[PtCl_4]$ was prepared by the well known reduction of the $K_2[PtCl_6]$ with hydrazine [29]. Solvents were obtained commercially and used without further purification. *(S,S)*-ethylendiamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid dihydrochloride and its methyl, ethyl and *n*-propyl esters, used for the synthesis of complexes described below, were prepared according to the procedure described in the literature [20] and were marked as $\mathbf{L^2}$, $\mathbf{L^3}$, $\mathbf{L^4}$, in the same manner as in our previous work. According to this, previously synthesized complex Pt(IV) with ligand $\mathbf{L^3}$, is designated as **C3** in this paper.

2.1.2. Methods

Elemental analyses were carried out with Elemental Vario EL III microanalyser. Infrared spectra were recorded on a Nicolet 6700 FTIR spectrometer using ATR technique. The NMR spectra were recorded on a Varian Gemini 200 instrument. Chemical shifts for ^{1}H and ^{13}C spectra were referenced to residual ^{1}H and ^{13}C presented in deuterated dimethylsulfoxide (DMSO). Melting points were determined on Electrothermal melting point apparatus.

Agilent 6210 Time-of-Flight LC/MS system (Agilent Technologies, Santa Clara, California, USA) equipped with an ESI interface (ESI–TOF–MS) was used for the identification of compounds. The ESI was operated in a positive mode and nitrogen was used as the drying gas (12 L/min) and nebulizing gas at 350 °C (45 psi). The OCT RF voltage was set to 250 V and the capillary voltage was set to 4.0 kV. The voltages applied to the fragmentor and skimmer were 140 V and 60 V respectively. Scanning was performed from 100 to 2000 *m/z* (mass-to-charge ratio). The compound identification was as follows: the compound was dissolved in the methanol (concentration of 1 mg/mL), and a direct injection of 0.01 μL sample was conducted by 1200 Series HPLC (Agilent Technologies, Waldbronn, Germany) without a separation column. The isocratic mobile phase consisted of 50% acetonitrile and 50% of 0.2% formic acid in water (v/v) at a flow rate of 0.2 mL/min.

2.1.3. Synthesis of $[PtCl_2(L^2)]$, C2a. Solution of ligand $\mathbf{L^2}$ (112.56 mg, 0.24 mmol) in methanol, neutralized with the solution of LiOH (c = 0.096 mol/dm^3, 5 mL) was added to a solution of $K_2[PtCl_4]$ (100 mg, 0.24 mmol) in H_2O (10 mL). The reaction mixture was stirred for 24 h at room temperature. The precipitate was filtered off and dried in vacuum. The compound was isolated as yellow powder.

Yield: 0.055g, 34.61%. Mp. 184 ºC. FTIR (ATR): $\tilde{\nu}$ (cm^{-1}) = 2928.0, 2852.7, 1744.5, 1651.0, 1447.5, 1219.1, 850.7. ^{1}H NMR (500 MHz, [D6]DMSO): 0.80 (m, C5, C5' 8H), 1.20 (m, C7, 4H), 1.40 (m, C4, 2H), 1.60 (m, C3, 4H), 1.80 (m, C6, C6' 8H), 2.20 – 2.40 (m, –NH–C***H*****$_2$**C***H*****$_2$**–NH–, 4H), 3.60 – 3.90 (m, C***H*****$_3$**–OOC–, 6H), 4.35 and 3.20 (m, –OOC–C***H***–NH–, 2H), 6.30 – 7.80 ppm (m, N***H***, 2H). ^{13}C NMR (50 MHz, [D6]DMSO): 25.47 (**C7**), 26.04 (**C6, C6'**), 33.70 (**C5, C5'**), 34.40 (**C4**), 35.78 (**C3**), 46.58 (**C8**), 51.24 and 52.24 (–O**C**H$_3$), 58.91 (**C2**), 170.25 ppm (**C1**). MS (LC/MS, 4000 V): m/z: $[M+NH_4^+]^+$ 679.23. Anal. Calcd. for $C_{22}H_{40}O_4N_2Cl_2Pt$ %: N 4.23; C 39.88; H 6.04. Found: N 4.20; C 39.49; H 6.31.

2.1.4. Synthesis of [$PtCl_2(L^3)$], C3a. The complex was synthesized as described for complex **C2a** using 119.28 mg (0.24 mmol) of $\mathbf{L^3}$. The compound was isolated as yellow powder.

Yield: 0.086 g, 51.93%. Mp. 168 ºC. FTIR (ATR): $\tilde{\nu}$ (cm^{-1}) = 2928.6, 2852.6, 1740.4, 1651.3, 1446.9, 1198.5, 840.9 cm^{-1}. ^{1}H NMR (500 MHz, [D6]DMSO): 0.80 – 1.50 (m, C7, 6',6, 12H; C$\boldsymbol{H_3}$$CH_2$OOC– 6H), 1.20 and 2.00 (m, C3, 4H; C5',5, 8H; C4, 2H; CH_3C$\boldsymbol{H_2}$–OOC–, 4H); 2.32 – 2.80 (m, CH_3C$\boldsymbol{H_2}$–OOC–, 4H), 3.94 – 4.15 (m, –NH–C$\boldsymbol{H_2}$C$\boldsymbol{H_2}$–NH–, 4H; –OOC–C$\boldsymbol{H}$–NH–, 2H), 6.61 – 7.07 ppm (d, N$\boldsymbol{H}$, 2H).^{13}C NMR (50 MHz, [D6]DMSO): 14.38 (**C**H_3CH_2OOC–), 26.50 (**C7**), 30.70 (**C6, C6'**), 32.40 (**C5, C5'**), 33.40 (**C4**), 34.50 (**C3**), 49.03 (**C8**), 59.34 and 60.33 (CH_3**C**H_2OOC–), 61.42 (**C2**), 170.14 and 171.28 ppm (**C1**). MS (LC/MS, 4000 V): m/z [M +NH_4^+]$^+$ 707.27. Anal. Calcd. for $C_{24}H_{44}O_4N_2Cl_2Pt{\cdot}H_2O$: N 4.00; C 41.20; H 6.44. Found: N 4.22; C 40.92; H 6.27.

2.1.5. Synthesis of [$PtI_2(L^2)$], C2b. KI (200 mg, 1.2 mmol) was added to a solution of $K_2[PtCl_4]$ (100 mg, 0.24 mmol) in H_2O (10 mL), and stirred for 10 minutes. A solution of ligand $\mathbf{L^2}$ (112.56 mg, 0.24 mmol) in methanol, primarily neutralized with $LiOH{\cdot}H_2O$ (20.14 mg, 0.48 mmol), was then added. The reaction mixture was stirred for 2 h at room temperature. The precipitate was filtered off and dried in vacuum. The compound was isolated as intensive yellow powder.

Yield: 0.096 g, 47.34%. Mp. 178 ºC. FTIR (ATR): $\tilde{\nu}$ (cm^{-1}) = 2927.3, 2851.6, 1742.9, 1650.4, 1446.1, 1206.9, 847.5 cm^{-1}. ^{1}H NMR (500 MHz, [D6]DMSO): 0.80 – 2.20 (m, C7**,** 4H; C5,5',6,6'**,** 8H; C3, 4H; C4, 2H) 2.60 – 3.00 (m, –NH_2–C$\boldsymbol{H_2}$C$\boldsymbol{H_2}$–NH_2–, 4H), 3.60 – 4.00 (m, C$\boldsymbol{H_3}$–OOC–, 6H), 4.20 – 4.80 (m, –OOC–C$\boldsymbol{H}$–NH_2–, 2H), 6.40 – 7.50 ppm (m, N$\boldsymbol{H}$, 2H). ^{13}C NMR spectrum (50 MHz, [D6]DMSO): 25.82 (**C7**), 26.17 (**C6, C6'**), 32.20 (**C5, C5'**), 33.07 (**C4**), 33.76 (**C3**), 46.24 (**C8**), 51.68 – 53.19 (–O**C**H_3), 58.24 (**C2**), 170.08 i 171.38 ppm (**C1**). MS (LC/MS, 4000 V): m/z: [M+NH_4^+]$^+$ 863.11. Anal. Calcd. for: $C_{22}H_{40}O_4N_2I_2Pt$: N 3.31; C 31.24; H 4.73. Found: N 3.68; C 30.90; H 5.12.

2.1.6. Synthesis of [$PtI_2(L^3)$], C3b. The complex was synthesized as described for complex **C2b** using 119.28 mg (0.24 mmol) of $\mathbf{L^3}$. The compound was isolated as intensive yellow powder.

Yield: 0.104 g, 62.80%. Mp. 162 ºC. FTIR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3095.0, 2925.1, 2851.1, 1741.1, 1633.7, 1446.1, 1194.2, 848.0 cm^{-1}. ^{1}H NMR (500 MHz, [D6]DMSO): 0.90 – 1.26 (m, C7, 6',6, 12H; C***H*****$_3$**CH_2OOC– 6H) 1.60 – 2.30 (m, C3, 4H; C5',5, 8H; C4, 2H; CH_3C***H*****$_2$**–OOC–, 4H), 2.77(m, CH_3C***H*****$_2$**–OOC–, 4H), 4.00 – 4.50 (m, –NH–C***H*****$_2$**C***H*****$_2$**–NH–, 4H; –OOC–C***H***–NH–, 2H), 6.40 – 7.50 ppm (m, N***H***, 2H). ^{13}C NMR (50 MHz, [D6]DMSO): 14.40 (**C**H_3CH_2OOC–), 26.15 (**C7**), 32.38 (**C6, C6'**), 33.55 (**C5, C5'**), 34.32 (**C4**), 36.46 (**C3**), 48.85 (**C8**), 53.03 (CH_3**C**H_2OOC–) 61.43 (**C2**), 171.04 ppm (**C1**). MS (LC/MS, 4000 V): m/z [M +NH_4^+]$^+$ 891.14. Anal. Calcd. for: $C_{24}H_{44}O_4N_2I_2Pt$: N 3.21; C 33.00; H 5.04. Found: N 3.36; C 33.43; H 5.08.

2.1.7. Synthesis of [PtI_2(L^4)], C4b. The complex was synthesized as described for complex **C2b** using 123.60 mg (0.24 mmol) of **L^4**. The compound was isolated as intensive yellow powder.

Yield: 0.117 g, 54.11%. Mp. 152 ºC. FTIR (ATR): $\tilde{\nu}$ (cm^{-1}) = 3155.7, 2926.1, 2852.5, 1740.8, 1648.5, 1448.0, 1189.9, 843.2 cm^{-1}. ^{1}H NMR (500 MHz, [D6]DMSO): 0.88 – 1.40 (m, C***H***$_3CH_2$–, 6H; C7, 2H, C6,C6', 8H), 1.55 – 1.90 (m, C7, 2H; C5,5', 8H; CH_3C***H*****$_2$**–, 4H), 1.90 – 2.15 (C3, 4H; C4, 2H) 2.60 – 3.00 (m, –C***H*****$_2$**–OOC–, 4H), 4.00 – 4.80 (m, –OOC–C***H***–NH–, 2H, –NH–C***H*****$_2$**C***H*****$_2$**–NH–, 4H), 6.50 – 7.50 ppm (m, N***H***, 2H).^{13}C NMR (50MHz, [D6]DMSO): 10.73 (**C**$H_3CH_2CH_2OOC$–), 21.87 (CH_3**C**H_2CH_2OOC–), 25.91 (**C7**), 32.42 (**C6, C6'**), 33.68 (**C5, C5'**), 34.30 – 35.08 (**C4**), 37.14 (**C3**), 49.50 and 52.12 (**C8**), 60.43 and 61.08 (CH_3CH_2**C**H_2OOC–), 67.25 and 67.80 (**C2**), 170.29 and 171.18 ppm (**C1**). MS (LC/MS, 4000 V): m/z [M+NH_4^+]$^+$ 919.17. Anal. Calcd. for: $C_{26}H_{48}O_4N_2I_2Pt$: N 3.11; C 34.63; H 5.33. Found: N 3.15; C 34.80; H 5.23.

2.2. Antitumor assessment

2.2.1. Cell cultures and reagents

Human cell lines: promyelocytic (HL-60) and lymphocytic leukemia (REH), glioma (U251) and lung carcinoma (H460), were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom). All reagents used in biological experiments were obtained from Sigma (St. Louis, MO, USA) unless stated otherwise. Cell lines were maintained at 37°C in a humidified atmosphere with 5 % CO_2, in HEPES (20 mM) buffered RPMI 1640 cell culture medium supplemented with 5 % fetal calf serum (10 % fetal calf

serum was used for leukemic cell lines), 2 mM L-glutamine, 10 mM sodium pyruvate, 1 % penicillin/streptomycin mixture. U251 and H460 cells were prepared for experiments by using the conventional trypsinisation procedure with trypsin/EDTA and incubated in 96-well flat-bottom plates (1×10^4 cells per well) for cell viability assessment. The cells were allowed to rest for 24 h before treatment. Leukemic cell lines HL-60 and REH were incubated in 96-well flat-bottom plates (3×10^4 cells/well) for the viability assessment or 24-well plates (2.5×10^5 cells/well) for the flow cytometry analysis (all plates were from Sarstedt, Nümbrecht, Germany). The leukemic cells were treated 2 h after seeding. All investigated cell lines were treated with novel Pt(II) complexes and corresponding ligands previously dissolved in DMSO according to their solubility and kept at room temperature until use. Each experiment contained two controls: untreated cells and cells treated with maximal concentration of DMSO used, in order to ensure that DMSO alone did not affect cell viability.

2.2.2. Cell viability

The acid phosphatase and the lactate dehydrogenase (LDH) assays were used to measure the number of viable cells.

The acid phosphatase assay is based on *p*-nitrophenyl phosphate hydrolysis by intracellular acid phosphatases in viable cells and subsequent production of *p*-nitrophenol. In brief, *p*-nitrophenyl phosphate solution (for HL-60 and REH: 55 mg p-nitrophenyl phosphate, 5 ml of 0.3 M sodium acetate buffer, pH 5.5, with 0.3% Triton X-100; for U251 and H460: 37.4 mg p-nitrophenyl phosphate, 7 ml of 0.1 M sodium acetate buffer, pH 5.5, with 0.1% Triton X-100) was added to each well and the samples were incubated for 2 h at 37°C. The reaction was stopped by addition of 50 µl of NaOH (1.3 M for HL-60 and REH and 1 M for U251 and H460) and the absorbance, which is directly proportional to the cell number, was measured in an automated microplate reader (Sunrise; Tecan, Dorset, UK) at 405 nm. The results were presented as % absorbance relative to untreated control. The IC_{50} values were calculated using GraphPad Prism software.

The release of cytosolic enzyme LDH reflects a loss of membrane integrity and therefore necrotic type of cell death. The test was performed exactly as previously described [30]. The results were calculated using formula: (E–C)/(T–C)x100, where E is the experimental

absorbance of treated cells, C is the control absorbance of untreated cells, and T is the absorbance of Triton X-100-lysed cells. The results were presented as a percentage (%) of viable cells.

2.2.3. Flow cytometric analysis of ROS production and apoptotic parameters

The flow cytometry analysis was performed using CellQuest Pro software for acquisition and analysis on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Production of reactive oxygen species (ROS) was determined by measuring the intensity of green fluorescence emitted by a non-selective redox-sensitive dye dihydrorhodamine 123 (DHR; Invitrogen, Paisley, UK), which was added to cell cultures (5 μM) at the beginning of treatment. After incubation, cells were washed in PBS and the increase in green fluorescence (FL1) was analyzed as a measure of ROS production. Mitochondrial production of superoxide ions was analyzed using flow cytometry by measuring the intensity of red fluorescence (FL2) emitted by a superoxide-specific fluorochrome dihydroethidium, DHE (Sigma-Aldrich), which was incubated with cells (20 μM) for 30 min at the end of the treatment. The type of cell death (apoptotic or necrotic) was analyzed by double staining with annexinV-fluorescein isothiocyanate (FITC), which binds to early apoptotic cells with exposed phosphatidylserine, and propidium iodide (PI), which labels the late apoptotic/necrotic cells with membrane damage (staining kit from BD Pharmingen, San Diego, CA). The annexin negative (Ann^-)/PI^+ cells were considered necrotic.

DNA fragmentation was assessed by the flow cytometric analysis of ethanol-fixed cells stained with DNA-binding red fluorescent (FL2 channel) dye propidium iodide (PI). The hypodiploid cells (sub-G_0 compartment) were considered apoptotic.

The activation of caspases was assessed using flow cytometer by measuring the increase in green fluorescence (FL1) of the cells stained with FITC-conjugated: pan-caspase (ApoStat; R&D Systems); caspase 8 (CaspGLOW Fluorescein Active Caspase-8 Staining Kit), 9 (CaspGLOW Fluorescein Active Caspase-9 Staining Kit) and 3 (CaspGLOW Fluorescein Active Caspase-3 Staining Kit) inhibitors, all from BioVision, Inc, Milpitas, California, exactly according to the manufacturer's instructions.

The acidic autophagolysosomes were detected by the flow cytometric analysis of the cells stained with pH-sensitive supravital dye acridine orange which stains cytoplasmic autophagolysosomes orange-red, while nuclei green. Therefore, if present, autophagy is

detected as an increase in red/green fluorescence ratio (FL3/FL1). After incubation, the cells were washed with PBS and stained with acridine orange (1 μM) for 15 min at 37°C. Cells were then washed in PBS and analyzed on the flow cytometer.

2.2.4. Statistical analysis

Statistical analyses were performed using Student's t-test for small independent samples. A p value of less than 0.05 ($p<0.05$) was considered significant.

3. Results and discussion

3.1. Synthesis of complexes

Complexes [$PtCl_2$**L**^{2}] and [$PtCl_2$**L**^{3}] (**C2a** and **C3a** respectively) were synthesized by the reaction of $K_2[PtCl_4]$ and corresponding ligand (**L**^{2}= dimethyl-(*S*,*S*)-ethylenediamine-*N*,*N'*-di-2-(3-cyclohexyl)propanoate and **L**^{3}= diethyl-(*S*,*S*)-ethylenediamine-*N*,*N'*-di-2-(3-cyclohexyl)propanoate) in molar ratio 1:1. In the synthesis of complexes [PtI_2L^2], [PtI_2L^3] and [PtI_2L^4] (**C2b, C3b** and **C4b** respectively) excess of potassium iodide was added to solution of $K_2[PtCl_4]$, and then corresponding ligand (**L**^{4}= di-*n*-propyl-(*S*,*S*)-ethylenediamine-*N*,*N'*-di-2-(3-cyclohexyl)propanoate) was added. Reactions were performed at room temperature in water/methanol solutions (Scheme 1). Complexes precipitated after 2 to 24 hours of stirring, in moderate yields. Complexes **C2b, C3b** and **C4b** are soluble in acetone, chloroform and DMSO while complexes **C2a** and **C3a** are soluble in DMSO.

3.2. Spectroscopic studies

In the IR spectra of all synthesized complexes characteristic C=O stretching bands appear at 1740 – 1745 cm^{-1}. There are no significant changes in the values for these vibrations compared to free ligands [20], indicating non-involvement of these groups into coordination. Characteristic bands for C–O stretching vibrations appear in the range from 1189 to 1219 cm^{-1}. Characteristic asymmetric C–N bands at 888.3 cm^{-1} were detected in spectra of all complexes. The absorption bands at around 2900-3100 cm^{-1} correspond to val(N-H) vibrations in secondary amines. These bands prove that ligands are deprotonated and

coordinated *via* nitrogen atoms to the central metal ion. (see the Supporting information, Fig.S1 – Fig.S5)

In the ^{1}H NMR spectra, the signals corresponding to cyclohexyl and alkyl protons from ester moiety (**R**CH_2O–) are in the range from 0.80 ppm to 2.20 ppm. From 2.60 to 3.00 ppm are chemical shifts for protons from ethylendiamine bridge, while signals attributable to protons (O)C–C**H**–NH are slightly shifted downfield (4.00 – 4.80 ppm) comparing to free ligands. Signals of RC**H_2**–OOC– protons were found at 3.60 – 4.00 ppm, similar to that of free ligands [19]. Signals of N**H** protons in complexes are shifted downfield corresponding to free ligands, and appear at 6.40 – 7.50 ppm. ^{13}C NMR spectra showed characteristic signal for carbonyl groups at 169 – 171 ppm. Signals attributable to carbon atoms from alkyl chains and cyclohexyl groups were detected in the range 10 – 37 ppm, while signals for –HN–**C**H_2–**C**H_2–NH–, R**C**H_2–OOC– and (O)C–**C**H–NH carbon atoms were found in the region from 47 to 67 ppm. (see the Supporting information, Fig.S6 – Fig.S20)

The mass spectra were recorded in positive mode and showed $[M+NH_4]^+$ ions, for all complexes.

3.3. Antitumor activity studies

Acid phosphatase assays demonstrated that both ligands and corresponding novel chlorido and iodido Pt(II) complexes reduced the number of viable cells in most of investigated tumor cell lines. Novel complexes had superior antitumor action compared to their organic ligands, with iodido being more efficient than chlorido complexes of the same type. With the exception of **C2a**, the efficacy of complexes was equal to or superior than cisplatin in 24-hour antitumor action. In this respect, **C3b** was the most powerful with IC_{50} value of approximately 3 μM on all tested tumor cell lines. Furthermore, the antileukemic action of **C3a** was 4 times stronger than its previously reported Pt(IV) complex analogue **C3** [20], or cisplatin (**Table 1**).

The antileukemic action of cyclohexyl analogues, esters (**L^2**–**L^5**) of ethylenediamine dipropanoic acid (**L^1**) has been previously reported, with **L^3** marked as the most potent ligand and the HL-60 cell line as the most sensitive to its cytotoxic action [31]. In the present study, human promyelocytic cell line (HL-60) was also the most sensitive to antitumor action of all investigated substances. Accordingly, we were particularly interested in determining the mode of antileukemic action of novel **C3a** and **C3b** complexes. Furthermore, antitumor

action of Pt(IV) chlorido complexes corresponding to herein investigated Pt(II), revealed good antiglioma action [20] but antileukemic action remained unexplored. Thus our goal was to examine the antileukemic potential of **C3**, chlorido Pt(IV) complex analogue, and compare it to chalogenido Pt(II) complexes (**C3a and C3b**) .

3.4. ROS production and induction of oxidative stress

Having in mind that oxidative stress was the initial event involved in antileukemic action of organic ligand $\mathbf{L^3}$ [31] we first analyzed the ability of novel Pt(II) complexes to increase production of ROS and cause oxidative stress in HL-60 cells.

Obtained results show that 4-hour treatment with both novel complexes, **C3a** and **C3b**, as well as Pt(IV) analogue **C3**, induced ROS production (when applied in IC_{50} concentrations) (Figure 2), unlike cisplatin and organic ligand $\mathbf{L^3}$, in the same experimental setting (data not presented for clarity). Namely, both **C3a** (5 μM) and **C3b** (3 μM) induced ROS hyperproduction with **C3b** being more potent, causing 2-fold increase in ROS-mediated DHR-derived fluorescence intensity (FL1) in comparison with untreated control (Figure 2A). Furthermore, **C3a** also caused (Figure 2B) the increase in DHE-derived fluorescence intensity (FL2), suggesting the superoxide hyperproduction indicative of oxidative stress. The treatment with **C3** complex (4h, 18 μM) caused more pronounced increase in DHR-derived FL1 fluorescence compared to novel complexes (Figure 2A), , suggesting that **C3,** despite the lack of noticeable increase in superoxide production (Figure 2B), induces rapid and prominent oxidative damage in HL-60 cells, as observed previously in U251 glioma cells [20]. On the other hand, it was shown that organic ligand induced mitochondria-derived superoxide hyperproduction in HL-60 cells [31], unlike cisplatin [20], indicating that cyclohexyl functionalized *edda* ligands significantly contributed to mitochondria-derived oxidative damage.

3.5. Investigation of cell death type

We next investigated the type of leukemic cell death induced by treatment with representative novel Pt(II) complexes (**C3a**, **C3b**) and their Pt(IV) complex analogue (**C3**).

The LDH test indicated that 24 h treatment with **C3a** (24h, 5 μM) and **C3b** (24h, 3 μM) resulted in less cell membrane damage than treatment with **C3** (24 h; 18 μM), suggesting that for novel Pt(II) complexes necrosis is not as relevant a mechanism of cell death as for Pt(IV) complex (Figure 3A)**.** This result is in accordance with the result obtained for **C3** on U251 glioma cells [20].

In order to determine the exact apoptotic pathway we investigated the activity of initiator caspases 8 and 9 and executioner caspase 3 in HL-60 cells after treatment. Novel complexes **C3a** and **C3b** both induced marked caspase 8 activation after 2 h, that decreased at later time points (6 h and 12 h) (Figure 3B). Complex **C3** also caused caspase 8 activation, but to a lesser extent compared to novel Pt(II) halogenido complexes. Unlike caspase 8, the increase in caspase 9 activity with novel Pt(II) halogenido complexes was moderate (1.7-fold after 2 h treatment) and also decreased during the time, whereas with **C3** caspase 9 activation was not detected (data not shown). Caspase 3 activation was more pronounced following novel Pt(II) halogenido complexes treatment (12 h), compared to the **C3** treatment (Figure 3C). Pan-caspase activation was still present in HL-60 cells after 24 h treatment with **C3a**, **C3b** and **C3** (Figure 3D). Our data show that novel Pt(II) halogenido complexes induce activation of both caspase 8 and 9, the former twice more pronounced than the latter. Although mixed (extrinsic-intrinsic) apoptotic HL-60 cell death has been described as a cytotoxic mechanism of action for some plant extracts and their chemical constituents [32], and taking into account a possible overlap in specificity of caspase inhibitors used [33], our data speak in favor of dominant role of caspase 8 activation in induction of leukemic cell death. The lack of caspase 9 activation by Pt(IV) analogue C3 implicates the major role of extrinsic apoptotic pathway.

Additionally, both novel complexes, **C3a** (5 μM) and **C3b** (3 μM), later (24 h) caused the increase in percentage of Ann^+ cells from 6.2 % in untreated control to > 50 % following treatment. On the other hand, **C3** treatment (24 h; 18 μM), caused not only the increase in percentage of Ann^+ cells, but also in necrotic (Ann^-/PI^+) cells (> 30 % in comparison to 1.0 % in control). The obtained results suggest the importance of apoptosis in leukemic cell death induced by novel Pt(II) complexes, while both apoptosis and necrosis apparently contribute to leukemic cell death induced by corresponding Pt(IV) complex (Figure 3C).

Furthermore, 24 h treatment of HL-60 cells with **C3a** (5 μM) and **C3b** (3 μM) caused massive disturbance in HL-60 cell cycle distribution with cell accumulation in sub-G_0 phase (> 40%) indicating DNA fragmentation (data not shown). The intrinsic AIF-mediated

leukemic cell death has been reported as organic $\mathbf{L^3}$ ligand mechanism of action [31]. The obtained results demonstrated that Pt(II) complexes investigated herein induce apoptotic cell death of leukemic cells in the concentration several times lower compared to organic ligands. This indicates that complexation with platinum improves the antileukemic effect and possibly contributes to the extrinsic apoptotic pathway induction. On the other hand, Pt(IV) complexation results in presence of both apoptotic and necrotic markers of leukemic cell death, but in higher concentrations in comparison with Pt(II) complexes. Having in mind that Pt(IV) complexes are reduced to Pt(II) complexes in bloodstream [34,35] in order to achieve their biologically active form, Pt(II) complexation with this type of organic ligands might be a better option for achieving antileukemic effect.

It is noteworthy that the treatment with **C3a** (5 μM) , **C3b** (3 μM) and **C3** (18 μM) failed to cause any increase in acidic cytoplasmic content in HL-60 cells (data not shown) implying the lack of autophagy involvement in novel Pt(II) complexes' antileukemic action and confirming the result obtained on U251 glioma cells treated with **C3** [20].

4. Conclusion

Herein we present the synthesis of new cisplatin analogues, dihalogenido-Pt(II) complexes with cyclohexyl substituted ethylendiamine type ligands and report better antitumor efficacy of iodido than corresponding chlorido complexes. The novel Pt(II) complexes, excluding **C2a**, exhibited comparable or better antitumor action in comparison with cisplatin and superior to precursor ligands. Human promyelocytic cell line (HL-60) was allocated as the most sensitive to cytotoxic action of all investigated substances, and was used for investigation of the underlying mode of antileukemic action. The investigated novel Pt(II) complexes **C3a** and **C3b** showed more potent antileukemic action than corresponding Pt(IV) complex **C3**, through induction of oxidative stress and apoptosis, evidenced by caspase activation and phosphatidylserine externalization. Obtained results indicate that novel Pt(II) halogenido complexes with cyclohexyl *edda* type ligands could have better antileukemic potential than corresponding Pt(IV) complexes and warrant further research.

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Conflict of interest

The authors hereby declare no conflict of interest.

Supporting Information

IR and NMR (^{1}H, ^{13}C, HMBC) spectra of synthesized complexes.

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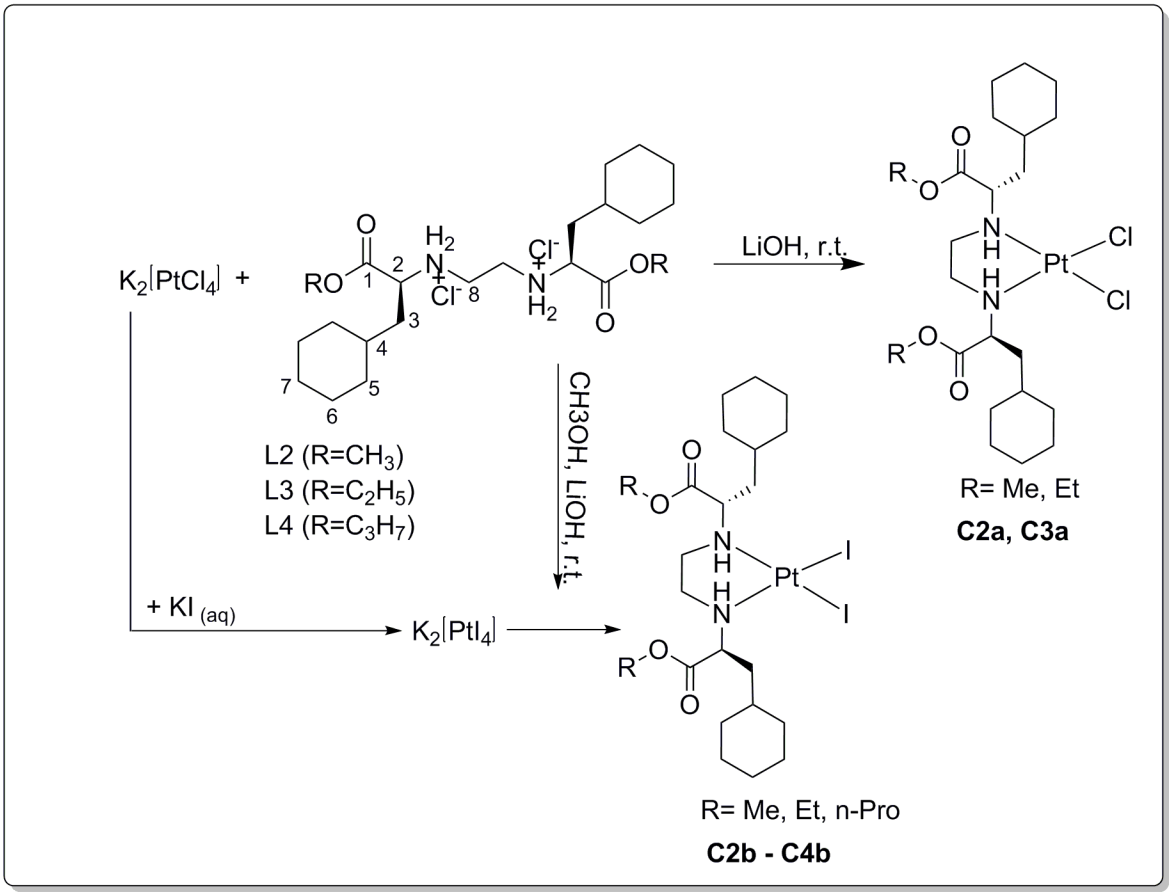
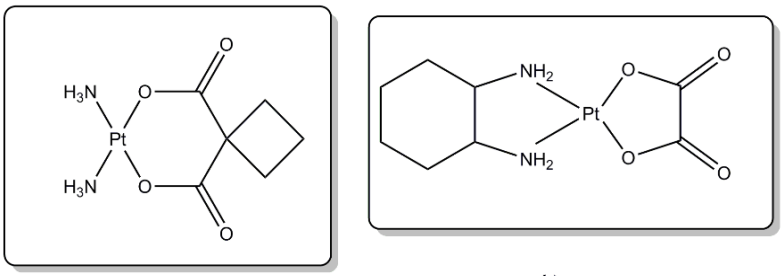


Table 1. *In vitro* cytotoxicity of organic compounds (**L**^{2}, **L**^{3}, **L**^{4}) and corresponding Pt(II) complexes determined by acid phosphatase assay after 24 h incubation.

Compound/ complex	IC_{50} (μM)			
	H460	U251	HL-60	REH
L^{2}	>100	40.2 ± 4.3	19.8 ± 1.3	73.6 ± 2.0
C2a	30.2*$^{\#}$ ± 3.2	7.0*$^{\#}$ ± 0.6	6.7*$^{\#}$ ± 0.4	9.1*$^{\#}$ ± 0.2
C2b	6.9*$^{\#}$ ± 0.5	4.5*$^{\#}$ ± 1.3	3.6*$^{\#}$ ± 0.2	6.5*$^{\#}$ ± 0.3
L^{3}	66.3 ± 2.7	26.6 ± 4.0	10.7 ± 1.4	44.4 ± 10.0
C3†	n.a	n.a	17.8 ± 1.2	n.a
C3a	16.4* ± 1.0	4.1*$^{\#}$ ± 0.1	3.8*$^{\#}$ ± 0.4	7.1*$^{\#}$ ± 0.7
C3b	3.5*$^{\#}$ ± 0.1	2.1*$^{\#}$ ± 0.2	2.1*$^{\#}$ ± 0.2	2.7*$^{\#}$ ± 0.3
L^{4}	49.7 ± 6.4	41.8 ± 8.2	80.8 ± 9.1	92.7 ± 13.3
C4b	8.6* ± 0.4	4.3*$^{\#}$ ± 0.3	2.7*$^{\#}$ ± 0.1	6.2*$^{\#}$ ± 0.2
cisplatin	10.4 ± 1.3	20.2 ± 2.9	17.5 ± 2.4	17.1 ± 1.6

Values represent the mean ± SD from two independent experiments. † - Pt (IV) analogue of C3a; *$p < 0.05$ denotes lower IC_{50} value compared to ligands; #$p<0.05$ denotes lower IC_{50} compared to cisplatin. n.a= not assessed



a) b)