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Novel methylene modified cyclohexyl ethylenediamine-*N*,*N*′-diacetate ligands and their platinum(IV) complexes. Influence on biological activity

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ABSTRACT

This paper focuses on the synthesis, characterization and biological activity of new *N*,*N*′-methylene modified cyclohexyl ethylenediamine-*N*,*N*′-diacetate (edda)-type ligands and their Pt(IV) complexes. Both the ligands and complexes were characterized by infrared, UV–vis, ESI-MS, 1D (^{1}H, ^{13}C, ^{195}Pt) and 2D (COSY, HSQC, HMBC) NMR spectroscopy and elemental analysis. The possible correlation between the reduction potentials and the cytotoxicity of the complexes was examined. The potential antitumoral activity of all compounds was tested *in vitro* on human melanoma A375, human glioblastoma U251, human prostate cancer PC3, human colon cancer HCT116, mouse melanoma B16 and mouse colon cancer CT26CL25 cells, as well as primary fibroblasts and keratinocytes. The results obtained revealed strong antitumor potential of the newly synthesized drugs with preserved efficacy against cisplatin resistant lines and less toxicity towards nonmalignant counterparts. The mechanism found to be responsible for the observed tumoricidal action of each synthesized compound was induction of apoptosis generally accompanied with caspase activation. Taken together, the effective response to the treatment of a wide range of different cell lines, including cisplatin resistant subclones, as well as induction of apoptosis, as the mechanism suggested to be the most desirable way of eliminating malignant cells, represents a great advantage of this novel group of drugs in comparison to other members in this metallo-drug family.

1. Introduction

Much interest in developing metal based drugs appeared in the mid-1960s [1]. As frequently used anticancer agents, platinum containing compounds have a unique place in metal coordination chemistry [2,3]. Almost five decades after Rosenberg's discovery of cisplatin [4,5], significant progress has been made in alleviating the cytotoxic properties of these compounds. In order to overcome severe side effects, such as nephrotoxicity, neurotoxicity, ototoxicity, nausea, *etc.* [6], numerous platinum based complexes have been synthesized [7]. Although the search for ideal anticancer agents continues, only two cisplatin analogues (carboplatin, oxaliplatin) [8] have led to progress in cancer treatment [9]. Rigorous clinical trials eliminated other potential drugs, such as ipro- and tetraplatin, so intensive research in this field continues. So far, satraplatin has shown the most beneficial results, although it is still undergoing active clinical trials [10]. The influence of complex geometry plays a great role in specific interactions leading to biological effects [11]. Platinum occurs in two oxidation states. The Pt(II) square planar complexes show several limitations compared with Pt(IV) octahedral complexes, such as less kinetic inertness, cis geometry and low coordination number [12]. Indisputable structural differences between Pt(II) and Pt(IV) complexes enable more lipophilic compounds to be obtained [13], which can be confirmed by greater cellular uptake and increased cytotoxicity [14]. The use of orally administrable anticancer drugs is desirable for numerous reasons (noninvasive therapies, reduced hospital costs), which are the main indications for synthesizing novel complexes.

Considerable progress has been made using axial ligands with several advantages [15], involving specific prodrug targeting and combination with bioactive compounds [16,17]. Selecting amine ligands [18–20] that are biologically active themselves, made a visible breakthrough in this field [21,22]. Ethylenediamine-*N*,*N*′-diacetate (edda)-type of ligands [23], together with their derivatives exhibit structure–activity relationships based on numerous *in vitro* tests [24]. While Pt(II) and Pt(IV) complexes with edda type ligands show significant cytotoxicity but are less efficient than cisplatin [25], Pt(IV) complexes linked to esterified edda type ligands have greater anticancer activity than cisplatin [16]. Moreover, intensive research has indicated two

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different mechanisms of cell death. The above mentioned Pt(IV) complexes with esterified edda type ligands induce rapid death by necrosis [26], while cisplatin causes slower apoptotic cell death [27].

Structure–activity relationships describe various structural changes in the amine ligands. Earlier investigations by Keppler and co-workers evaluated the way that modifying ligands influences the cytotoxic properties [18,28]. Hence, the main concept of our study was to examine whether methylation *via* both nitrogen donor atoms of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid affects the biological activity of the corresponding ligands and complexes. In this work, the synthesis, characterization and biological activity of Pt(IV) complexes with methylated cyclohexyl functionalized edda-type ligands are reported. In addition, their biological activity was evaluated through assessment of cell viability and subsequent basic analysis of the mechanism of drug action. The results presented here reveal the strong antitumor potential of these compounds evident even in cell lines resistant to conventional cisplatin. Finally, in comparison to non-methylated forms of esterified edda type Pt(IV) complexes, the novel drugs act through induction of apoptosis followed by caspase activity.

2. Experimental

2.1. Chemistry

2.1.1. Starting agents

The precursor substances, methyl, ethyl, *n*-propyl and *n*-butyl esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid were synthesized starting from (*S*)-2-amino-3-cyclohexylpropanoic acid hydrochloride purchased from Senn Chemicals (Dielsdorf, Switzerland). The entire method was previously described and published [26]. $K_2[PtCl_6]$ was synthesized by the standard procedure [29].

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. ^1H and ^{13}C spectra were obtained using a Varian Gemini 200 spectrometer, while Burker Avance III 500 spectrometer was employed for 1D (^1H, ^{13}C), 2D COSY (Correlation Spectroscopy) and 2D ^1H–^{13}C heteronuclear correlation spectra using $CDCl_3$ and TMS as the reference. The ^{195}Pt NMR spectra were recorded on a Varian Unity 400 NMR spectrometer and the shifts were determined using hexachloroplatinic acid as external standard (δ 0 ppm). Recording ESI spectra was noted on a 6210 Time-of-Flight LC–MS instrument (G1969A, Agilent Technologies) in methanol. A GBC UV/Vis Cintra 6 spectrometer was used for electronic spectra of complexes dissolved in methanol at 1×10^{-4} M. Reagents and solvents were of commercial reagent grade quality and used without further purification. An electrothermal melting point apparatus was used for determining the melting points of each complex. 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 glassy carbon electrode (Model 6.1204.300), 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, C1–C4 were dissolved in DMSO in concentrations of 1.0 mM containing $LiClO_4$ as supporting electrolyte.

2.2. Biology

2.2.1. Reagents and cells

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), DMSO 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), collagenase IV from *Clostridium hystoliticum*, DNase, and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). Annexin V-FITC (AnnV) was from Biotium (Hayward, CA). Tested compounds were dissolved in DMSO and kept at −20 °C. The U251 glioblastoma cell line was a kind gift from Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The B16 murine melanoma was a kind gift from Dr. Siniša Radulović (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia). Human melanoma A375, colon cancer HCT116 and SW620, prostate PC3 and mouse colon CT26CL25 cell lines were a kind gift from Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.01% sodium pyruvate and antibiotics (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2. For the experiments, cells were detached by standard trypsinization, resuspended in culture medium and seeded at 1×10^4/well in 96-well plates for viability determination and 2.5×10^5/well in 6-well plates for flow cytometry.

2.2.2. Preparation of primary cell cultures of keratinocytes and adult lung fibroblasts

Primary keratinocyte cell culture was prepared from mouse ear skin. Briefly, excised ears were rinsed in 70% ethanol, washed in PBS and split, the skin was spread dermis side down in a sterile tissue culture dish and left to float on 0.5% trypsin-PBS solution for 1 h at 37 °C. The epidermis was removed, cut into small pieces and transferred to a conus tube. Cells were vigorously resuspended to obtain single cell suspensions and filtered through nylon mesh. Cells were washed twice to remove trypsin, and finally resuspended in 15% FCS-RPMI and immediately used for experiments. The primary fibroblast cell culture was prepared from adult mouse lungs. In brief, lungs were aseptically removed, rinsed in PBS, cut into small pieces and incubated in collagenase IV from *Clostridium histolyticum* (0.7 mg/ml) in addition to DNase (30 μg/ml) with slow stirring for 30 to 90 min at 37 °C. The solution containing tissue fragments was centrifuged, the pellet was washed three times and resuspended in 15% FCS-RPMI. After incubation for 24 h in the tissue culture dish, nonadherent cells and tissue fragments were removed and the medium was replaced. At 80–90% confluence, the cells were split and used for experiments.

2.2.3. Cell viability evaluation by crystal violet and MTT assay

Cell viability was estimated colorimetrically by crystal violet (CV) assay for adherent cells, or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for nonadherent cells. Tumor cell lines (1×10^4/well) were treated for 24 h with a broad range of drug concentrations, whereas lung fibroblasts (3×10^4/well) and keratinocytes (8×10^4/well) were treated for 24 h with IC50 doses of each drug. For the CV assay, at the end of treatment cells were rinsed with PBS, fixed for 10 min with 4% paraformaldehyde and stained with 2% crystal violet-PBS for 15 min at room temperature. The absorbance of dye dissolved in 33% acetic acid, which correlates with the number of viable cells, was measured in a microplate reader at 540 nm, while the background was measured at 640 nm. For nonadherent cells, MTT was added to cell suspensions in a final concentration of 0.5 mg/ml. After 2 h incubation at 37 °C cells were collected, centrifuged and pellets were dissolved in DMSO. The absorbance, which correlates with the level of mitochondrial-dependent MTT reduction to formazan, was measured in a microplate reader at 540 nm with the reference wavelength at 640 nm. The results are presented as a percentage of the value for untreated cells (control) which was arbitrarily set at 100%.

2.2.4. Cell cycle analysis

After treatment with the IC50 dose of each compound for 24 h, cells were fixed for 45 min with 70% ethanol at 4 °C, washed twice with cold PBS and incubated with 20 μg/ml PI and 0.1 mg/ml

RNase for 30 min at 37 °C in the dark. Quantification of cellular DNA stained with red fluorescent PI was analyzed on a FACS Calibur flow cytometer (BD, Heidelberg, Germany) using a peak fluorescence gate to discriminate aggregates, while cell distribution in cell cycle phases was determined with Cell Quest Pro software (BD) [30].

2.2.5. AnnexinV-FITC/PI staining

Apoptotic cells, which are characterized by phosphatidylserine exposed to the extracellular environment while cell membrane integrity remains intact, were detected using an annexinV-FITC/PI staining kit (Biotium, Hayward, CA). Cells were treated for 14 h with IC50 doses of each compound, trypsinized and stained according to the manufactureur's instructions. Cells were analyzed on a FACS Calibur flow cytometer using Cell Quest Pro software.

2.2.6. Caspase detection

For detection and quantification of caspase activity in apoptotic cells ApoStat (R&D Systems, Minneapolis, MN USA) was used. Briefly, cells were treated with IC50 doses of selected compounds for 24 h, collected and stained with ApoStat in phenol red free RPMI supplemented with 5% FCS for 15 min at 37 °C. After washing, cells were analyzed on a FACS Calibur flow cytometer using Cell Quest Pro software.

2.2.7. Statistical analysis

The results are presented as mean ± SD of triplicate observations from one representative of at least three experiments with similar results, except if indicated otherwise. The significance of the differences between various treatments was assessed by ANOVA followed by the Student Newman–Keuls test. $P<0.05$ was considered to be significant.

2.3. Synthesis of the ligands

2.3.1. Synthesis of the dimethyl ester of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)propanoic acid, L1

A suspension of the precursor, methyl ester of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (0.2 g, 0.43 mmol) in methanol (10 ml) was mixed and heated up to 40 °C for 20 min on a steam bath until the mixture was well homogenized. The methylation mixture was made by dissolving sodium triacetoxyborohydride (0.27 g, 1.29 mmol) in 10 ml methanol followed by adding 36% aqueous formaldehyde (0.13 ml, 4.3 mmol). The obtained solution for methylation was poured dropwise into the previously made suspension. In order to adjust the pH value to 4–5, glacial acetic acid (0.25 ml) was slowly added to the reaction mixture. The next portion of the same volume was added after 2 h and stirring was continued for the following 30 min. The whole amount of the reaction solution was washed out with diethylether (40 ml) followed by rinsing the ether extract with three equal portions (10 ml) of 1 M KOH and a 10 ml portion of brine. The combined ether solutions were dried overnight using anhydrous K_2CO_3 and evaporated *in vacuo* next day to obtain a colorless, oily liquid. Total yield: 98 mg, 56.31%. Anal. Calc. for $C_{23}H_{40}N_2O_4{\cdot}0.25CH_3OH$ (*M*r = 416.58): C, 67.03; H, 9.92; N, 6.72%; Found: C, 66.77; H, 9.98; N, 6.82%. ν_{max}(ATR)/cm^{-1}: 2923, 2850, 1734, 1683, 1448, 1193, 1161; ESI-MS (methanol), positive: *m*/*z* 397.34 [M-CH_2 + 3H]$^+$. ^{1}H NMR (500.26 MHz, $CDCl_3$) δ_H 0.89 (m, C5a′, C5b′, 4H), 1.08–1.34 (m, C7′, C6′, C4, 8H), 1.52 (m, –C*H*$_{2'}$–Cy, 2H), 1.59–1.71 (m, –C*H*$_2$–Cy, C7, C5a, C6, 10H), 1.76 (br d, C5b, 2H), 2.85 (m, –N–CH_2–C*H*$_2$–N–, 2H), 2.98 (m, –N–C*H*$_2$–CH_2–N–, 2H), 3.39 (dd, –OOC–C*H*–N–, 2H), 3.62 (s, –N–C*H*$_2$–N–, 2H), 3.70 (s, C*H*$_3$OOC–, 6H); ^{13}C NMR (125.80 MHz, $CDCl_3$); δ_C 26.10 (C6), 26.44 (C7), 33.12 (C5b), 33.47 (C5a), 34.32 (C4), 38.59 (–C*H*$_2$–Cy), 48.07 (–N–C*H*$_2$–C*H*$_2$–N–), 51.31 (C*H*$_3$OOC–), 61.82 (–OOC–C*H*–N–), 69.42 (–N–C*H*$_2$–N–), 173.49 (CH_3OOC–).

2.3.2. Synthesis of the diethyl ester of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)propanoic acid, L2

A suspension of the precursor, ethyl ester of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (0.2 g, 0.40 mmol) in methanol (10 ml) was mixed and heated up to 40 °C for 20 min on a steam bath until the mixture was well homogenized. The methylation mixture was made by dissolving sodium triacetoxyborohydride (0.25 g, 1.2 mmol) in 10 ml methanol followed by adding 36% aqueous formaldehyde (0.12 ml, 4.0 mmol). The obtained solution for methylation was poured dropwise into the previously made suspension. In order to adjust the pH value to 4–5, glacial acetic acid (0.25 ml) was slowly added to the reaction mixture. The next portion of the same volume was added after 2 h and stirring was continued for the following 30 min. The whole reaction solution was washed out with diethylether (40 ml) followed by rinsing the ether extract with three equal portions (10 ml) of 1 M KOH and a 10 ml portion of brine. The combined ether solutions were dried overnight using anhydrous K_2CO_3 and evaporated *in vacuo* the next day to obtain a colorless, oily liquid. Total yield: 103 mg, 58.68%. Anal. Calc. for $C_{25}H_{44}N_2O_4{\cdot}0.75CH_3OH$ (*M*r = 460.66): C, 67.14; H, 10.28; N, 6.08%; Found: C, 66.88; H, 10.77; N, 6.32%. ν_{max}(ATR)/cm^{-1}: 2920, 2848, 1727, 1681, 1446, 1155, 1098; ESI-MS (methanol), positive: *m*/*z* 425.35 [M-CH_2 + 3H]$^+$. ^{1}H NMR (500.26 MHz, $CDCl_3$) δ_H 0.82 (m, C5a′, C5b′, 4H), 1.01–1.28 (m, C7′, C6′, C*H*$_3$$CH_2$OOC–, C4, 14H), 1.45 (m, –C*H*$_{2'}$–Cy, 2H), 1.52–1.64 (m, –C*H*$_2$–Cy, C7, C5a, C6, 10H), 1.70 (br d, C5b, 2H), 2.79 (m, –N–CH_2–C*H*$_2$–N–, 2H), 2.93 (m, –N–C*H*$_2$–CH_2–N–, 2H), 3.30 (dd, –OOC–C*H*–N–, 2H), 3.57 (s, –N–C*H*$_2$–N–, 2H), 4.10 (m, CH_3C*H*$_2$–OOC–, 4H); ^{13}C NMR (125.80 MHz, $CDCl_3$) δ_C 14.31 (C*H*$_3$$CH_2$OOC–), 26.10 (C6), 26.43 (C7), 33.07 (C5b), 33.52 (C5a), 34.33 (C4), 38.63 (–C*H*$_2$–Cy), 48.05 (–N–C*H*$_2$–C*H*$_2$–N–), 60.15 (CH_3C*H*$_2$OOC–), 61.90 (–OOC–C*H*–N–), 69.38 (–N–C*H*$_2$–N–), 172.98 (CH_3CH_2OOC–).

2.3.3. Synthesis of the dipropyl ester of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)propanoic acid, L3

A suspension of the precursor, *n*-propyl ester of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (0.2 g, 0.38 mmol) in methanol (10 ml) was mixed and heated up to 40 °C for 20 min on a steam bath until the mixture was well homogenized. The methylation mixture was made by dissolving sodium triacetoxyborohydride (0.24 g, 1.14 mmol) in 10 ml methanol followed by adding 36% aqueous formaldehyde (0.11 ml, 3.8 mmol). The obtained solution for methylation was poured dropwise into the previously made suspension. In order to adjust the pH value to 4–5, glacial acetic acid (0.25 ml) was slowly added to the reaction mixture. The next portion of the same volume was added after 2 h and stirring was continued for the following 30 min. The whole reaction solution was washed out with diethylether (40 ml) followed by rinsing the ether extract with three equal portions (10 ml) of 1 M KOH and a 10 ml portion of brine. The combined ether solutions were dried overnight using anhydrous K_2CO_3 and evaporated *in vacuo* the next day to obtain a colorless, oily liquid. Total yield: 98 mg, 55.42%. Anal. Calc. for $C_{27}H_{48}N_2O_4{\cdot}0.5CH_3OH$ (*M*r = 480.70): C, 68.71; H, 10.48; N, 5.83%; Found: C, 69.09; H, 10.97; N, 6.06%. ν_{max}(ATR)/cm^{-1}: 2924, 2851, 1733, 1682, 1450, 1258, 1165; ESI-MS (methanol), positive: *m*/*z* 453.37 [M-CH_2 + 3H]$^+$. ^{1}H NMR (199.97 MHz, $CDCl_3$) δ_H 0.74–1.02 (m, C5a′, C5b′, C*H*$_3$$CH_2$$CH_2$OOC–, 10H), 1.03–1.40 (m, C7′, C6′, C4, 8H), 1.42–1.85 (m, –C*H*$_{2'}$–Cy, –C*H*$_2$–Cy, CH_3C*H*$_2$$CH_2$OOC–, C7, C5a, C6, C5b, 18H), 2.80–3.08 (m, –N–C*H*$_2$–C*H*$_2$–N–, 4H), 3.39 (dd, –OOC–C*H*–N–, 2H), 3.65 (s, –N–C*H*$_2$–N–, 2H), 4.05 (t, CH_3CH_2C*H*$_2$OOC–, 4H); ^{13}C NMR (50.28 MHz, $CDCl_3$) δ_C 10.20 (C*H*$_3$$CH_2$$CH_2$OOC–), 21.74 ($CH_3$C*H*$_2$$CH_2$OOC–), 25.86 (C6), 26.18 (C7), 32.81 (C5b), 33.30 (C5a), 34.09 (C4), 38.46 (–C*H*$_2$–Cy), 47.81 (–N–C*H*$_2$–C*H*$_2$–N–), 61.62 (CH_3CH_2C*H*$_2$OOC–), 65.58 (–OOC–C*H*–N–), 69.12 (–N–C*H*$_2$–N–), 172.86 ($CH_3CH_2CH_2$OOC–).

2.3.4. Synthesis of the dibutyl ester of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)propanoic acid, L4

A suspension of the precursor, *n*-butyl ester of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (0.2 g, 0.36 mmol) in methanol (10 ml) was mixed and heated up to 40 °C for 20 min on a steam bath until the mixture was well homogenized. The methylation mixture was made by dissolving sodium triacetoxyborohydride (0.23 g, 1.08 mmol) in 10 ml methanol followed by adding 36% aqueous formaldehyde (0.11 ml, 3.6 mmol). The obtained solution for methylation was poured dropwise into the previously made suspension. In order to adjust the pH value to 4–5, glacial acetic acid (0.25 ml) was slowly added to the reaction mixture. The next portion of the same volume was added after 2 h and stirring was continued for the following 30 min. The whole reaction solution was washed out with diethylether (40 ml) followed by rinsing the ether extract with three equal portions (10 ml) of 1 M KOH and a 10 ml portion of brine. The combined ether solutions were dried overnight using anhydrous K_2CO_3 and evaporated *in vacuo* the next day to obtain a colorless, oily liquid. Total yield: 106 mg, 59.55%. Anal. Calc. for $C_{29}H_{52}N_2O_4 \cdot 0.5CH_3OH$ ($Mr = 508.75$): C, 69.64; H, 10.70; N, 5.51%; Found: C, 69.48; H, 10.90; N, 5.68%. ν_{max}(ATR)/cm^{-1}: 2925, 2851, 1730, 1680, 1450, 1252, 1163; ESI-MS (methanol), positive: *m/z* 481.40 $[M\text{-}CH_2 + 3H]^+$. ^1H NMR (199.97 MHz, $CDCl_3$) δ_H 0.78–1.01 (m, C5a′, C5b′, $CH_3CH_2CH_2CH_2OOC-$, 10H), 1.05–1.47 (m, C7′, C6′, $CH_3CH_2CH_2CH_2OOC-$, C4, $-CH_{2'}-$Cy, 14H), 1.49–1.86 (m, $-CH_2-$Cy, $CH_3CH_2CH_2CH_2OOC-$, C7, C5a, C6, C5b, 16H), 2.79–3.06 (m, $-N-CH_2-CH_2-N-$, 4H), 3.37 (dd, $-OOC-CH-N-$, 2H), 3.63 (s, $-N-CH_2-N-$, 2H), 4.08 (t, $CH_3CH_2CH_2CH_2OOC-$, 4H); ^{13}C NMR (50.28 MHz, $CDCl_3$) δ_C 13.64 ($CH_3CH_2CH_2CH_2OOC-$), 19.14 ($CH_3CH_2CH_2CH_2OOC-$), 26.11 (C6), 26.42 (C7), 30.64 ($CH_3CH_2CH_2CH_2OOC-$), 33.03 (C5b), 33.58 (C5a), 34.34 (C4), 38.71 ($-CH_2-$Cy), 48.11 ($-N-CH_2-CH_2-N-$), 61.98 ($CH_3CH_2CH_2CH_2OOC-$), 64.09 ($-OOC-CH-N-$), 69.44 ($-N-CH_2-N-$), 173.19 ($CH_3CH_2CH_2CH_2OOC-$).

2.4. Synthesis of the complexes

2.4.1. Synthesis of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)-dimethylpropanoato-tetrachlorido-platinum(IV), C1

A mixture of $K_2[PtCl_6]$ (0.12 g, 0.24 mmol) dissolved in 10 ml of warm water and an equimolar amount of the ligand, dimethyl ester of *N*,*N*′-methylene-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoic acid, (0.10 g, 0.24 mmol), dissolved in 10 ml of dichloromethane was stirred under reflux at 40 °C for 3 days. During that period, the whole amount of dichloromethane evaporated and the formed solid product was filtered off, rinsed with diethyl ether and air dried. The complex was obtained as an orange powder. Total yield: 86 mg, 47.13%. mp: 88 °C. Anal. Calcd for $C_{23}H_{40}N_2O_4PtCl_4 \cdot 0.5H_2O$ ($Mr = 754.47$): C, 36.61; H, 5.48; N, 3.71%; Found: C, 37.05; H, 5.97; N, 3.50%. ν_{max}(ATR)/cm^{-1}: 2924, 2851, 1742, 1634, 1444, 1271, 1218, 886; UV/Vis (CH_3OH): λ_{max} (ε, 1872 $M^{-1}\,cm^{-1}$): 207 nm; ESI-MS (methanol), positive: *m/z* 408.29 $[M\text{-}PtCl_4 + H]^+$. ^1H NMR (199.97 MHz, $CDCl_3$) δ_H 0.90 (m, C5a′, C5b′, 4H), 1.06–1.40 (m, C7′, C6′, C4, 8H), 1.42–2.00 (m, $-CH_{2'}-$Cy, $-CH_2-$Cy, C7, C5a, C6, C5b, 14H), 2.78–3.06 (m, $-N-CH_2-CH_2-N-$, 4H), 3.38 (dd, $-OOC-CH-N-$, 2H), 3.62 (s, $-N-CH_2-N-$, 2H), 3.70 (s, CH_3OOC-, 6H); ^{13}C NMR (50.28 MHz, $CDCl_3$); δ_C 22.59 (C6), 22.91 (C7), 29.54 (C5b), 30.02 (C5a), 30.82 (C4), 35.15 ($-CH_2-$Cy), 44.56 ($-N-CH_2-CH_2-N-$), 56.69 (CH_3OOC-), 58.42 ($-OOC-CH-N-$), 65.92 ($-N-CH_2-N-$), 169.56 (CH_3OOC-). ^{195}Pt NMR (85.98 MHz, $CDCl_3$): 145.6.

2.4.2. Synthesis of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)-diethylpropanoato-tetrachlorido-platinum(IV), C2

A mixture of $K_2[PtCl_6]$ (0.11 g, 0.23 mmol) dissolved in 10 ml of warm water and an equimolar amount of ligand, diethyl ester of *N*,*N*′-methylene-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid, (0.10 g, 0.23 mmol), dissolved in 10 ml of dichloromethane was stirred under reflux at 40 °C for 3 days. During that period, the whole amount of dichloromethane evaporated and the formed solid product was filtered off, rinsed with diethyl ether and air dried. The complex was obtained as an orange powder. Total yield: 89 mg, 50.24%. mp: 90 °C. Anal. Calcd for $C_{25}H_{44}N_2O_4PtCl_4 \cdot 0.5H_2O$ ($Mr = 782.52$): C, 38.37; H, 5.80; N, 3.58%; Found: C, 38.52; H, 6.29; N, 4.06%. ν_{max}(ATR)/cm^{-1}: 2922, 2849 1735, 1635, 1447, 1260, 1195, 851; UV/Vis (CH_3OH): λ_{max} (ε, 2802 $M^{-1}\,cm^{-1}$): 213 nm; ESI-MS (methanol), positive: *m/z* 436.07 $[M\text{-}PtCl_4 + H]^+$. ^1H NMR (199.97 MHz, $CDCl_3$) δ_H 0.74–1.02 (m, C5a′, C5b′, CH_3CH_2OOC-, 10H), 1.04–1.40 (m, C7′, C6′, C4, 8H), 1.42–1.86 (m, $-CH_{2'}-$Cy, $-CH_2-$Cy, C7, C5a, C6, C5b, 14H), 2.78–3.08 (m, $-N-CH_2-CH_2-N-$, 4H), 3.38 (dd, $-OOC-CH-N-$, 2H), 3.64 (s, $-N-CH_2-N-$, 2H), 4.07 (m, CH_3CH_2OOC-, 4H); ^{13}C NMR (50.28 MHz, $CDCl_3$) δ_C 10.40 (CH_3CH_2OOC-), 26.16 (C6), 26.38 (C7), 32.99 (C5b), 33.52 (C5a), 34.30 (C4), 38.69 ($-CH_2-$Cy), 48.05 ($-N-CH_2-CH_2-N-$), 61.91 (CH_3CH_2OOC-), 65.84 ($-OOC-CH-N-$), 69.41 ($-N-CH_2-N-$), 173.17 (CH_3CH_2OOC-). ^{195}Pt NMR (85.98 MHz, $CDCl_3$): 150.9.

2.4.3. Synthesis of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)-dipropylpropanoato-tetrachlorido-platinum(IV), C3

A mixture of $K_2[PtCl_6]$ (0.11 g, 0.22 mmol) dissolved in 10 ml of warm water and an equimolar amount of ligand, dipropyl ester of *N*,*N*′-methylene-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid, (0.10 g, 0.22 mmol), dissolved in 10 ml of dichloromethane were stirred under reflux at 40 °C for 3 days. During that period, the whole amount of dichloromethane evaporated and the formed solid product was filtered off, rinsed with diethyl ether and air dried. The complex was obtained as an orange powder. Total yield: 87 mg, 50.43%. mp: 93 °C. Anal. Calcd for $C_{27}H_{48}N_2O_4PtCl_4$ ($Mr = 801.569$): C, 40.46; H, 6.04; N, 3.49%; Found: C, 40.27; H, 6.35; N, 3.78%. ν_{max}(ATR)/cm^{-1}: 2925, 2850, 1738, 1640, 1448, 1242, 1198, 887; UV/Vis (CH_3OH): λ_{max} (ε, 1818 $M^{-1}\,cm^{-1}$): 208 nm; ESI-MS (methanol), positive: *m/z* 464.89 $[M\text{-}PtCl_4 + H]^+$. ^1H NMR (199.97 MHz, $CDCl_3$) δ_H 0.78–1.01 (m, C5a′, C5b′, $CH_3CH_2CH_2OOC-$, 10H), 1.02–1.40 (m, C7′, C6′, C4, 8H), 1.42–1.85 (m, $-CH_{2'}-$Cy, $-CH_2-$Cy, $CH_3CH_2CH_2OOC-$, C7, C5a, C6, C5b, 18H), 2.78–3.06 (m, $-N-CH_2-CH_2-N-$, 4H), 3.39 (dd, $-OOC-CH-N-$, 2H), 3.65 (s, $-N-CH_2-N-$, 2H), 4.07 (t, $CH_3CH_2CH_2OOC-$, 4H); ^{13}C NMR (50.28 MHz, $CDCl_3$) δ_C 10.20 ($CH_3CH_2CH_2OOC-$), 21.74 ($CH_3CH_2CH_2OOC-$), 25.85 (C6), 26.18 (C7), 32.80 (C5b), 33.30 (C5a), 34.09 (C4), 38.45 ($-CH_2-$Cy), 47.82 ($-N-CH_2-CH_2-N-$), 61.62 ($CH_3CH_2CH_2OOC-$), 65.59 ($-OOC-CH-N-$), 69.12 ($-N-CH_2-N-$), 172.85 ($CH_3CH_2CH_2OOC-$).

2.4.4. Synthesis of N,N′-methylene-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)-dibutylpropanoato-tetrachlorido-platinum(IV), C4

A mixture of $K_2[PtCl_6]$ (0.10 g, 0.20 mmol) dissolved in 10 ml of warm water and an equimolar amount of ligand, dibutyl ester of *N*,*N*′-methylene-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid, (0.10 g, 0.20 mmol), dissolved in 10 ml of dichloromethane was stirred under reflux at 40 °C for 3 days. During that period, the whole amount of dichloromethane evaporated and the formed solid product was filtered off, rinsed with diethyl ether and air dried. The complex was obtained as an orange powder. Total yield: 93 mg, 55.23%. mp: 96 °C. Anal. Calcd for $C_{29}H_{52}N_2O_4PtCl_4 \cdot 0.25H_2O$ ($Mr = 834.13$): C, 41.76; H, 6.34; N, 3.36%; Found: C, 41.47; H, 6.25; N, 3.85%. ν_{max}(ATR)/cm^{-1}: 2924, 2851, 1737, 1635, 1451, 1251, 1195, 887; UV/Vis (CH_3OH): λ_{max} (ε, 2160 $M^{-1}\,cm^{-1}$): 211 nm; ESI-MS (methanol), positive: *m/z* 492.38 $[M\text{-}PtCl_4 + H]^+$. ^1H NMR (199.97 MHz, $CDCl_3$) δ_H 0.78–1.00 (m, C5a′, C5b′, $CH_3CH_2CH_2CH_2OOC-$, 8H), 1.04–1.46 (m, C7′, C6′, $CH_3CH_2CH_2CH_2OOC-$, C4, $-CH_{2'}-$Cy, 14H), 1.49–1.88- (m, $-CH_2-$Cy, $CH_3CH_2CH_2CH_2OOC-$, C7, C5a, C6, C5b, 16H), 2.78–3.07 (m, $-N-CH_2-CH_2-N-$, 4H), 3.37 (dd, $-OOC-CH-N-$, 2H), 3.62 (s, $-N-CH_2-N-$, 2H), 4.10 (t, $CH_3CH_2CH_2CH_2OOC-$, 4H); ^{13}C NMR (50.28 MHz, $CDCl_3$) δ_C 13.63 ($CH_3CH_2CH_2CH_2OOC-$), 19.13 ($CH_3CH_2CH_2CH_2OOC-$), 26.11 (C6), 26.41 (C7),

30.63 ($CH_3CH_2CH_2CH_2OOC-$), 33.02 (C5b), 33.57 (C5a), 34.34 (C4), 38.71 ($-CH_2-Cy$), 48.10 ($-N-CH_2-CH_2-N-$), 61.97 ($CH_3CH_2CH_2CH_2$-OOC−), 64.09 ($-OOC-CH-N-$), 69.44 ($-N-CH_2-N-$), 173.18 ($CH_3CH_2CH_2CH_2OOC-$).

3. Results and discussion

3.1. Synthesis of ligands

The reductive methylation of methyl, ethyl, *n*-propyl and *n*-butyl esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid was typical [31] with slight modifications. Precursor esters were well homogenized in methanol. The mixture for methylation containing 36% aqueous formaldehyde and sodium triacetoxyborohydride dissolved in methanol, was slowly added to the ligand solution which was stirred for the following 2 h. The pH value was adjusted using glacial acetic acid. The obtained solution was treated with appropriate amounts of diethylether, 1 M KOH and brine. After drying ether solutions overnight, ligands were obtained by evaporation *in vacuo*. All ligands were soluble in methanol, ethanol, dimethyl sulfoxide, diethyl ether, acetone, dichloromethane and chloroform.

3.2. Synthesis of complexes

The prepared $K_2[PtCl_6]$ [29] dissolved in warm water was mixed with an equimolar amount of ligand solution in dichloromethane and stirred under reflux at 40 °C for 3 days. The corresponding complexes were filtered off, rinsed with diethyl ether and air dried. All products were obtained as an orange powder. The complexes were soluble in methanol, ethanol, dimethyl sulfoxide, acetone, dichloromethane and chloroform (Fig. 1).

3.3. Spectroscopic studies

IR spectra of the free ligands, L1–L4, showed characteristic strong bands around 1730 cm^{-1} [32,33], which correspond to the C═O group. As these bands could also be found in the IR spectra of the corresponding complexes around 1740 cm^{-1}, coordination of the carboxylic oxygen to the metal center is excluded. Asymmetric CH_2 stretching vibrations were found around 2920 and 2850 cm^{-1} in the form of two strong bands for both ligands and complexes. Characteristic bands for tertiary amines identified in the area between 1210 and 1150 cm^{-1} were confirmed in the IR spectra of all ligands and complexes. Due to the ligand coordination, C–N vibrations of complexes were shifted toward higher wavenumbers.

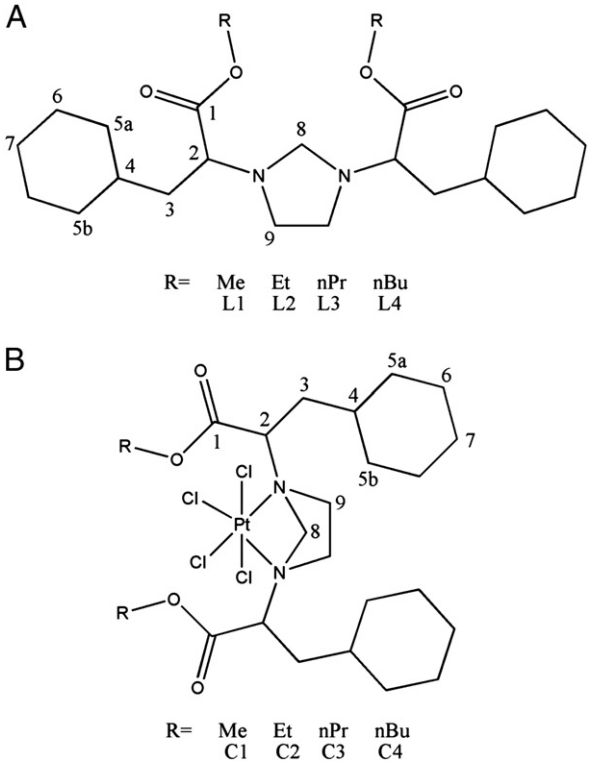

Fig. 1. A) Synthesized ligands L1–L4 and B) Pt(IV) complexes C1–C4.

Mass spectra of each ligand gave the strongest peak originating from the $[M\text{-}CH_2+3H]^+$ fragment and matched the calculated molecular mass. The observed fragmentation indicated that the formed $-N-CH_2-N-$ bond was the weakest one in ligands, L1–L4. On the other hand, mass spectra in positive mode for all complexes detected a $[M\text{-}PtCl_4+H]^+$ fragment as the most intensive peak [34,35].

^1H NMR spectra gave well-dissolved signals characteristic of a cyclohexyl moiety in the range 0.8–1.6 ppm for both ligands and complexes (Supplementary material Fig. S1–S4). Two sets of signals were observed for the $-CH_2-$(C5) from the cyclohexyl group, due to the diastereotopic nature of these protons induced by the chiral C atom. The shifts assignable to $-N-CH_2-CH_2-N-$ protons were found around 2.90 ppm in the form of two separated multiplets but were slightly moved to a lowered chemical shift in the spectra of complexes. The obvious doublet of doublets located around 3.40 ppm was related to (ROOC)CH–proton. All protons originating from alkyl groups of the complexes showed similar shifts to those of the free ligands. The central confirmation of the $-CH_2-$ group bonded to both nitrogen atoms was the singlet around 3.6 ppm in the ^1H NMR spectra of all ligands and complexes. The corresponding signal around 69 ppm in the ^{13}C NMR spectra and proper C–H correlation in the 2D NMR spectra are additional confirmation of the presence of the methylene bridge in ligand and complex structures. In the ^{195}Pt NMR spectra complexes C1 and C2 exhibit chemical shifts at 145 and 150 ppm, respectively. Thus, indicating on the octahedrally configured Pt(IV) complex with $[N_2, Cl_4]$ donor set [36].

Electronic spectra of all complexes showed a peak around 29 903 cm^{-1} (210 nm), which indicated $^1A_{1g}$–$^1T_{1g}$ *d*–*d* transitions (38 300–28 000 cm^{-1}) [37] and octahedral geometry of Pt(IV) complexes.

Based on the spectroscopic data, we confirmed octahedral geometry around the Pt(IV) center with *N*,*N*′ coordinated ligands and four chloride ions for all complexes.

3.4. Electrochemistry

The reduction potentials of the complexes, C1–C4, are in the range from −816 to −924 mV and the data are given in Table 1. The similar reduction potentials (−705 and −846 mV) were found for analog Pt(IV) complexes having R_2eddp (R = Et, *n*-Pr; eddp = ethylenediamine-*N*,*N*′-di-3-propionate) ligands [38]. The Pt(IV) to Pt(II) reduction is irreversible process followed by the loss of the chloride ligands [39,40]. As the length of the alkyl chain increase, small differences in *E*p values are observed (Supplementary material Fig. S5). Overall, the strong correlation between reduction potentials and biological activity was not detected.

3.5. Ligands, L1–L4 and complexes, C1–C4 strongly down-regulated tumor cell growth

To evaluate the potential antitumor effect of newly synthesized ligands and the corresponding Pt(IV) complexes, cells were treated with a broad range of doses for 24 h and viability was assessed by CV test. As shown in Fig 2, both ligands and complexes down-regulated the growth of different cell lines, such as human melanoma A375, human glioblastoma U251, human prostate cancer PC3, human colon cancer HCT116, mouse melanoma B16 and mouse colon cancer

Table 1
Reduction potential values (*vs.* Ag/AgCl) of the complexes C1–C4.

*E*p/mV	
C1	−816
C2	−900
C3	−884
C4	−924

CT26CL25. As expected, complexes C1–C4 were more efficient at reducing cell viability in comparison with the corresponding ligands. Thus, complexes reached their IC50 values at doses in which ligands were completely ineffective (Table 2). These values were even lower than those for the conventional cytostatic drug, cisplatin, in all cell lines tested. The activity of the ligands was influenced by the length of the alkyl chain and should range from L4, which was ineffective, to L1–2 whose activity was similar. However, the corresponding complexes were similarly cytotoxic except for C1, which displayed at least two-fold less potential in decreasing tumor cell viability. In comparison to the anticancer activity of non-methylated forms of esterified edda type of Pt(IV) complexes, IC50 values of the novel compounds were similar or slightly higher [26]. Moreover, drug activity was not limited to the type and origin of the tumor cell line and even occurred in cell lines resistant or poorly responsive to treatment with cisplatin, such as HCT116 and CT26CL25, respectively (Table 2). Therefore, these two cell lines were selected for further analysis.

3.6. Ligands, L1–L4 and complexes, C1–C4 were less toxic to primary cells

In order to determine the toxicity of drugs towards primary cells, mouse fibroblasts and keratinocytes were treated with the dose selected for each compound. This was the highest IC50 dose of each compound chosen from the list of IC50 doses determined on different tumor cells. Analysis of cell viability by MTT and CV tests after 24 h

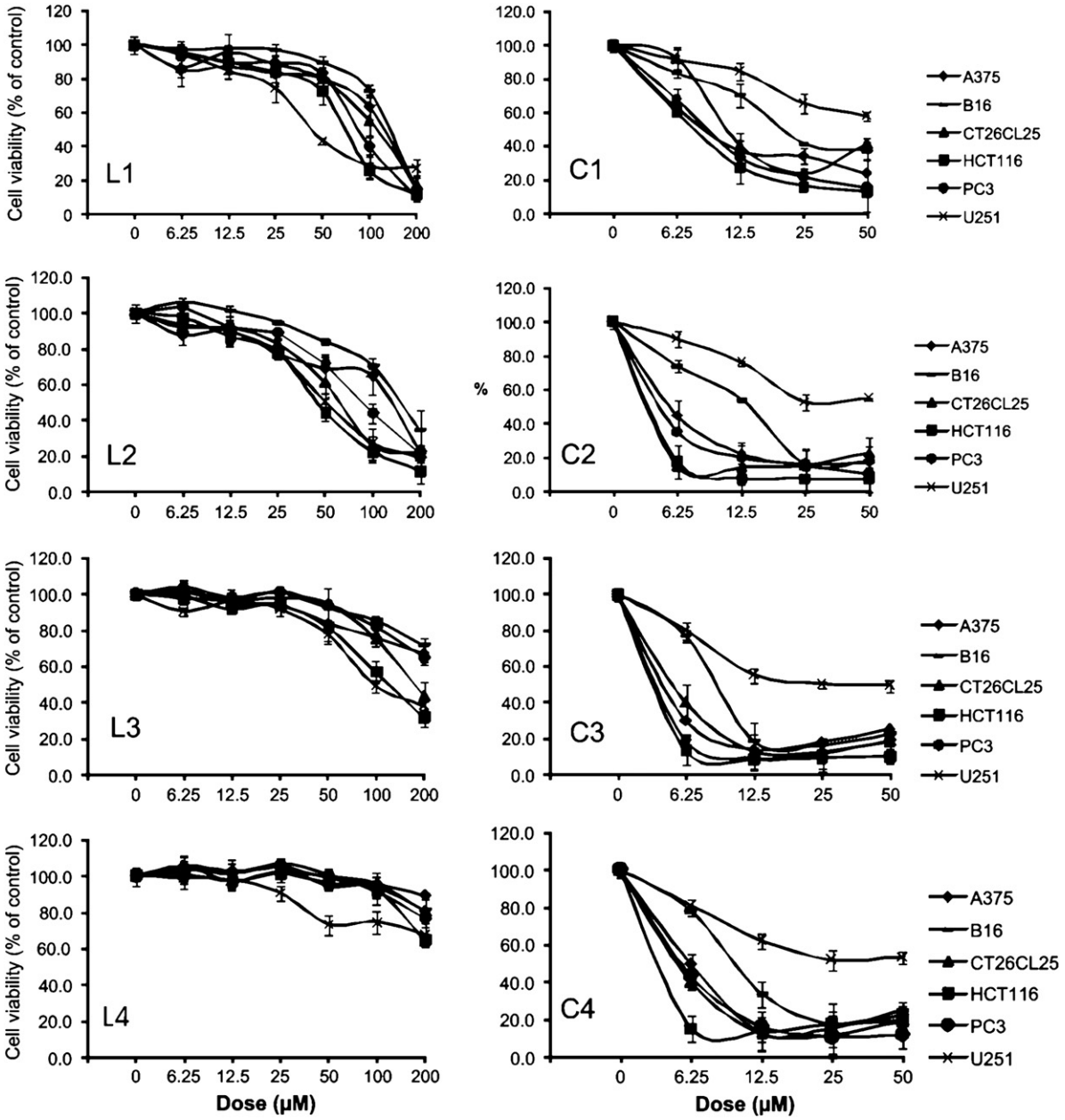

Fig. 2. The effect of ligands L1–L4 and complexes C1–C4 on viability of tumor cell lines. Cells (1×10^4/well) were treated with different doses of the tested compounds for 24 h, and cell viability was determined by CV assay. The data are presented as mean ± SD from a representative of three independent experiments.

Table 2
In vitro cytotoxicity of examined compounds determined by CV test. IC50 (μM) values are from representative of three independent experiments.

Cell line/ compound	Cisplatin	L1	L2	L3	L4	C1	C2	C3	C4
PC3	12.5	88.3	89.2	>200	>200	8.1	6.2	5.4	5.1
U251	20	44.1	51	98.3	>200	17.5	2.9	12.5	11.8
A375	45	127.3	134.8	>200	>200	9.1	7.2	5.7	6.2
B16	94.3	108.3	135.5	>200	>200	21.3	13.8	9.1	10.2
HCT116	>120	74.1	45.9	127.1	>200	8.8	4.1	6.3	5.9
CT26CL25	120	114	65.9	179.9	>200	11.3	5.2	6.3	6.3

revealed that the applied doses were significantly less toxic for primary keratinocytes and fibroblasts than for tumor cells (Fig 3). Except in the case of treatment with C2 and L3, the decrease of viability did not exceed 30% of control and was similar in both types of primary cells. In accordance with data in the literature, the IC50 dose of cisplatin obtained on keratinocytes and fibroblasts under the same conditions was approximately 30 μM. This is significantly lower than doses found to be efficient in tumor cell lines, underlining the great toxicity of cisplatin towards primary tissues [41,42]. Taken together, apart from strong tumoricidal potential the newly synthesized drugs affected the viability of normal cells to a lower extent.

3.7. Ligands, L1–L4 and complexes, C1–C4 induced caspase dependent apoptosis

Previous data showed that the synthesized ligands and their Pt(IV) complexes significantly diminished cell viability [43]. To explore further the cause of viability reduction upon drug treatment, the presence of different modes of cell death was examined in HCT116 and CT26CL25 cells, which are resistant to conventional

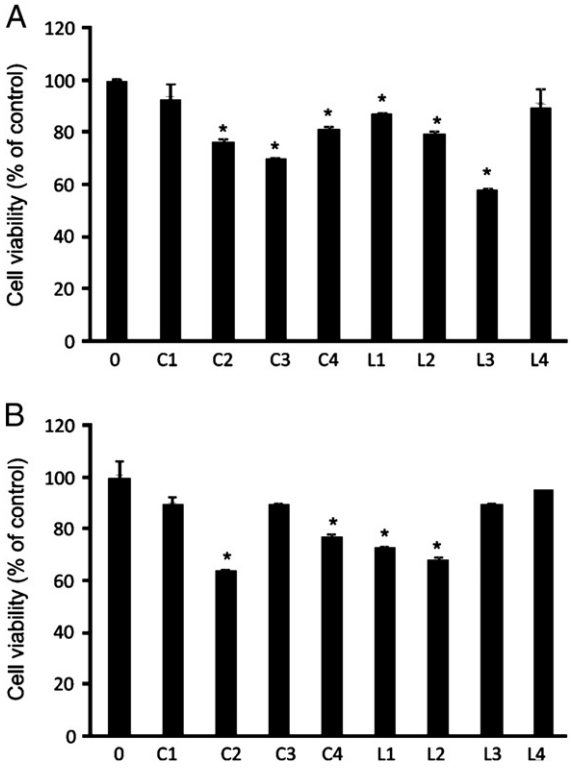

Fig. 3. The effect of ligands L1–L4 and complexes C1–C4 on viability of normal cells. (A) Fibroblasts (3×10^4/well) and (B) keratinocytes (8×10^4 cells/well) were treated for 24 h with 200 μM of L1–L4, 20 μM of C1 or 12.5 μM of C2–C4, after which cell viability was determined by CV and MTT assay, respectively. The data are presented as mean ± SD from a representative of three independent experiments. *P<0.05, refers to untreated cultures.

cisplatin treatment. L4 was excluded from further analysis because its IC50 dose was higher than 200 μM. Cell cycle distribution was analyzed first. As shown in Fig. 4A, the IC50 doses of both ligands and complexes elevated the percentage of cells in the sub G compartment after 24 h of treatment, which indicated accumulation of cells with fragmented DNA. In comparison to control cells where less than 5% of apoptotic cells were detected, there were 30% hypodiploid cells upon treatment with the experimental drugs. These data suggested that their mode of action is preferentially induction of apoptosis rather than cell cycle arrest and subsequent inhibition of proliferation. To delineate the apoptotic process, the cells were stained with Ann/PI 14 h after drug addiction. The results revealed significant accumulation of Ann^+ PI^- cells with inverted phosphatidylserine as a marker of early apoptosis (Fig. 4B) in all treatments applied. Subsequently, the amount of double Ann^+PI^+ positive cells was elevated. Cells accumulated in this compartment are marked as necrotic cells due to disturbed permeability or membrane rupture [44]. However, LDH release, as a marker of necrotic cell death, was not detected before 24 h of cultivation in the presence of the drugs, indicating that this path to death is not the primary target of drug action (not shown).

To assess the mechanism of the triggered apoptotic process, total caspase activity was determined in the treated cultures. In concordance with the observed apoptosis, caspase activation was observed in almost all treated cultures (Fig. 4C). The intensity of caspase activation varied significantly from treatment to treatment. Interestingly, the highest caspase activation was observed in cultures exposed to L2, L3, C3 and C4, which were found to be more toxic for primary cells. This indicated that the intensity of caspase activity could not be extrapolated with the rate of apoptosis. Thus, different tumor cells showed diverse sensitivities to apoptotic cell death not necessarily correlated with the intensity of caspase activation [45,46]. Moreover, apoptosis induction by L1 was not followed by caspase activation, suggesting that this type of cell death may be independent of caspase activation (Fig. 4C). To clarify this, HCT116 cells were exposed to L1 in the presence of 20 μM of ZVAD-fmk inhibitor for 24 h and viability was measured by CV test. The presence of this caspase inhibitor did not abolish the toxicity of L1 (viability 50 ± 7.6% in L1 treated cells *vs* 56 ± 3.1% in co-treatment) confirming the irrelevance of caspase activation.

Octahedral Pt(IV) complexes with the esterified edda type of ligands expressed antitumor potential similar to cisplatin but with different mechanisms involved. In contrast to cisplatin, these drugs triggered rapid necrotic death [24]. Necrotic cell death was described as accidental cell death, which promoted local inflammation and further tissue damage, as well as recruitment of immune inflammatory cells which support tumor progression through angiogenesis inside neoplastic tissue, cancer cell proliferation and invasiveness [47]. On the other hand, apoptosis finalized by consumption of apoptotic bodies by neighboring cells prevented an inflamed tumor microenvironment [24]. The more important presence of apoptotic debris stimulated dendritic cell maturation with subsequent priming of a desirable specific antitumor immune response [48]. In this light, the feature that methylation *via* both nitrogen donor atoms enables alkyl esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid to trigger apoptosis instead of necrosis is a great advantage of this family of drugs in comparison to earlier ones.

4. Conclusion

Platinum(IV) complexes derived from cyclohexyl edda derivatives are attracting attention as they allow a variety of substitutions favorable for potential anticancer drugs. In this context, we examined the influence of a $-CH_2-$ group introduced ethylenediamine chain of the cyclohexyl-edda precursor on spectroscopic and biological features of ligands and their corresponding complexes. The applied chemical intervention generated a novel class of molecules found to

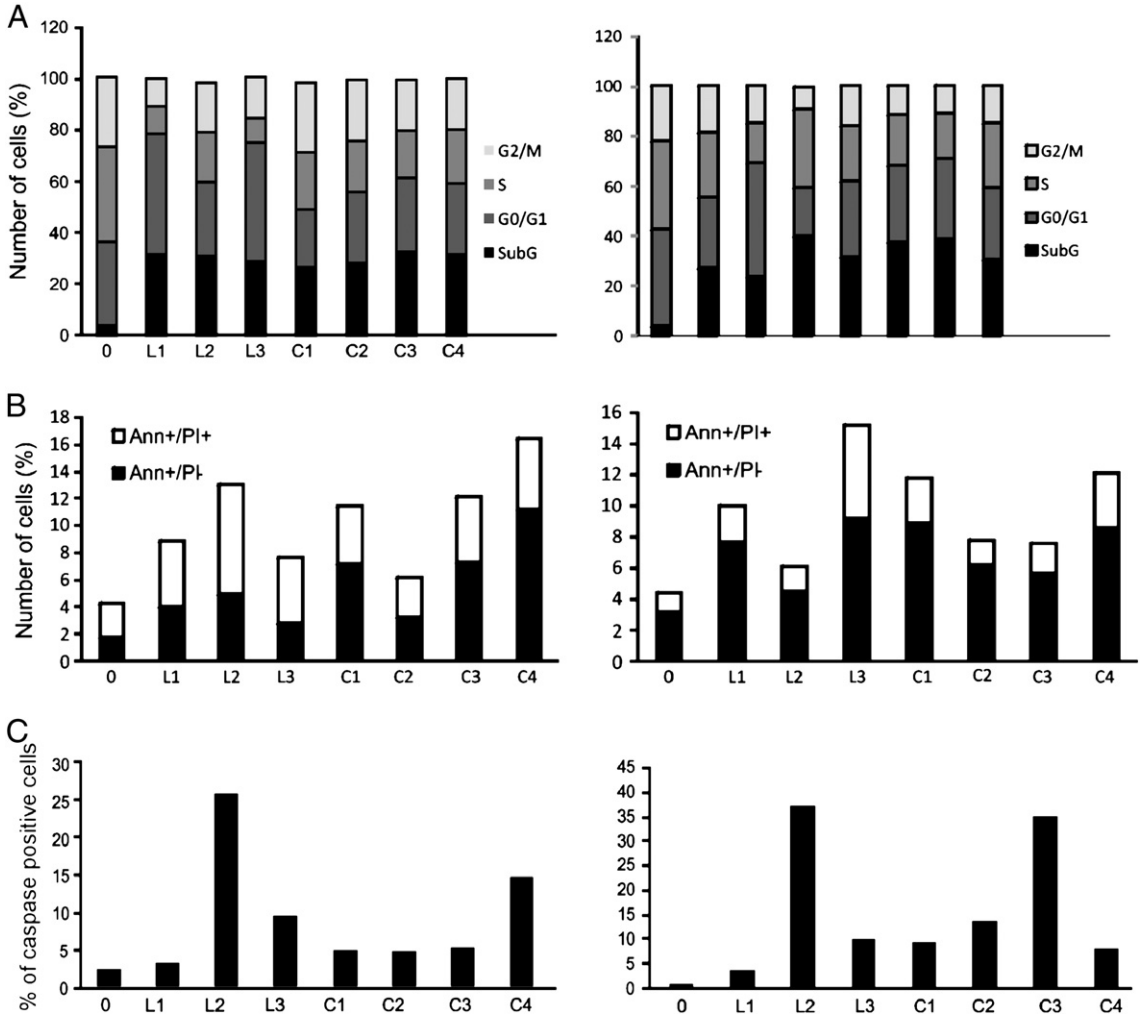

Fig. 4. Apoptotic cell death induced by ligands L1–L4 and complexes C1–C4. HCT116 (left panel) and CT26CL26 cells (right panel) (2.5×10^5/well) were exposed to the IC50 dose of each compound. Cell cycle distribution was assessed after 24 h (A), Ann/PI double staining after 14 h (B) and caspase activity after 24 h (C). Results from one of three representative flow cytometric analyses are presented.

be efficient against numerous cell lines of different specificity regarding their origin or the relevant signaling pathways involved in cancerogenesis (prostate, glioblastoma, melanoma, colon cancer). Moreover, their efficacy was not compromised by initial resistance to the original drug, cisplatin, as found for HCT116 and CT26CL25 colon cancer cells. On the other hand, primary keratinocytes and fibroblasts were less affected by treatment with the modified compounds indicating their selectivity toward cancer cells. Similarly to cisplatin, the novel compounds induced apoptosis of cancer cells but at a lower range of doses. Except in the case of L1, the induced apoptotic process correlated with caspase activation in all treatments. Apoptosis as the proposed mechanism of action, together with an intensified anticancer potential accompanied with less toxicity towards primary cells qualify the novel methylated forms of esterified edda type of Pt(IV) complexes and ligands as suitable candidates for further evaluation in this field.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2012.01.012.

Abbreviations

TMS	tetramethylsilane
ESI	electrospray ionization
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ANOVA	analysis of variance
CV	crystal violet

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