Journal of Organometallic Chemistry 749 (2014) 142–149

Contents lists available at ScienceDirect

Journal of Organometallic Chemistry

journal homepage: www.elsevier.com/locate/jorganchem

Synthesis, X-ray structure and strong *in vitro* cytotoxicity of novel organoruthenium complexes

Marija Mojić [a,1], Aleksandar Savić [b,1], Vladimir B. Arion [c], Mirna Bulatović [a], Jelena M. Poljarević [b], Djordje Miljković [a], Tibor J. Sabo [b], Sanja Mijatović [a], Danijela Maksimović-Ivanić [a,1], Sanja Grgurić-Šipka [b,*,1]

[a] *Department of Immunology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia*
[b] *Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia*
[c] *Institute of Inorganic Chemistry, University of Vienna, Währinger Strasse 42, A-1090 Vienna, Austria*

ARTICLE INFO

Article history:
Received 5 July 2013
Received in revised form
19 August 2013
Accepted 22 August 2013

Keywords:
Apoptosis
Cancer
Organoruthenium
Amine ligands

ABSTRACT

Two *p*-cymene ruthenium chlorido complexes containing isobutyl (**C1**) and isoamyl (**C2**) esters of (*S,S*)-ethylenediamine-*N,N′*-di-2-(3-cyclohexyl)propanoic acid as ligands were prepared from *p*-cymene ruthenium dichloride dimer and corresponding ester. All compounds have been characterized by elemental analysis, IR, ESI-MS, ^{1}H and ^{13}C NMR spectroscopy. Single crystal X-ray structure diffraction analysis of **C1** shows the usual piano-stool geometry of complexes, with coordination of ester ligand via nitrogen donor atoms. Ligands exhibit moderate anticancer activity ($IC_{50} > 50$ μM), while the complexes were significantly more cytotoxic towards various cancer cell lines, including B16, A375, HCT116, A549 and MCF7 cells (IC_{50} min.–max. 2.9–8.0 μM). We stress that cisplatin resistant HCT116 cell line was highly sensitive to the treatment with **C1** and **C2** (IC_{50} values: 4.4 and 5.5 μM *versus* $IC_{50} > 120$ μM for cisplatin). In parallel, primary fibroblasts-MRC-5 were remarkably less affected by these compounds.

1. Introduction

Cisplatin is still one of the most frequently used anticancer drugs [1]. It is mainly used in the treatment of ovarian, head and neck, bladder, cervical and lymphomas cancers [2]. However, there are some major drawbacks in the application of this drug: the efficiency for a limited range of cancers, severe side-effects, acquired or intrinsic resistance [3,4].

In the last years, complexes with metal ions other than platinum, e.g., gallium and ruthenium, have been investigated for their anticancer properties [5,6]. These compounds have a general lower toxicity and some of them (KP46, KP1019, NAMI-A) entered clinical trials with promising results [7,8].

During the last decade, the field of ruthenium arene complexes has received considerable attention as anticancer agents, due to their physicochemical properties that include chemical stability and structural diversity. Organoruthenium complexes of the type $[(\eta^6\text{-arene})Ru^{II}(XY)Z]^+$ (where XY is a chelating ligand, and Z is monoanionic ligand) are highly cytotoxic and *in vivo* reduce tumor growth. Hydrolysis of Ru–Z bond may be important step in the mechanism of cytotoxic action. The rate of hydrolysis of the Ru–Z bond is highly dependent on the nature of Z, if this group is labile, it can assure a faster hydrolysis and coordination site for biomolecules [9–11]. The arene ligand stabilizes the metal in its lower oxidation state and provides a hydrophobic face for the passive transport inside the cell [12–14]. The arene ligands are strongly coordinated to the ruthenium center and relatively inert towards substitution reactions. The three remaining coordination sites opposite to the π-ligand can be occupied with a wide variety of mono- or bidentate ligands with N-, O-, S- or P-donor atoms [15]. Unlike platinum(II) complexes which exhibit cytotoxicity by binding to DNA (in particular to the N7 atom of adjacent guanines), the mode of action of ruthenium anticancer drugs is still unclear [16,17]. Complexes bearing *N,N′*-chelating ligands have shown cytotoxicity comparable to that of cisplatin in a number of cell lines [18,19]. These ligands contain nitrogen atoms and are capable of stabilizing +2 oxidation state of ruthenium [20]. Structure activity

* Corresponding author. Studentski trg 12-16, 11000 Beograd, Serbia. Tel.: +381 113336736; fax: +381 112184330.
E-mail addresses: sanjag@chem.bg.ac.rs, sanja.grguric@gmail.com (S. Grgurić-Šipka).
[1] These authors equally contributed to this work.

0022-328X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jorganchem.2013.08.041

relationship for ethylenediamine-*N*,*N*′-diacetate (edda)-type of ligands is established according to numerous *in vitro* test, against a panel of various cancer cell lines [21,22]. This class of compounds includes different esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid. These compounds showed a distinct anti-tumor activity, with ethyl ester being the most active [23,24].

In this work, we describe the preparation, characterization and *in vitro* anticancer activity of the ruthenium(II) arene complexes with new esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid. The results presented herein reveal the strong antitumor activity of these complexes, even in cell lines resistant to conventional cisplatin. Particularly interesting is the fact that these ruthenium complexes are very active towards a variety of cell lines, while the ligands themselves do not show significant biological activity. Apoptosis as a type of programmed cell death induced by these complexes represents a major mechanism of their action.

2. Material and methods

2.1. Chemistry

$RuCl_3 \cdot 3H_2O$ was purchased from Johnson Matthey (London, United Kingdom). (*S*)-2-Amino-3-cyclohexyl-propanoic acid hydrochloride was purchased from Senn Chemicals (Dielsdorf, Switzerland). $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ [25] and (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid dihydrochloride [23] were prepared according to a published procedure, while corresponding esters were obtained as described in this paper. Solvents were obtained from commercial sources and used without further purification. Elemental analysis was carried out with Elemental Vario EL III microanalyzer. Infrared spectra were recorded on a Nicolet 6700 FT-IR spectrometer using ATR technique. The NMR spectra were recorded on a Bruker Avance III 500 spectrometer. Mass spectra were measured with a 6210 Time-of-Flight LC-MS instrument (G1969A, Agilent Technologies) by using DMSO/H_2O for ligands and acetonitrile as solvent for complexes. Electronic spectra of 1×10^{-3} M solutions of organometallic compounds in acetonitrile were recorded on a GBC UV/Vis Cintra 6 spectrophotometer. Melting points were determined on an Electrothermal melting point apparatus.

2.2. Biology

2.2.1. Reagents and cells

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). AnnexinV-FITC (AnnV) was from Biotium (Hayward, CA). ZVAD was from Promega (Madison, USA) while Apostat was bought from R&D (R&D Systems, Minneapolis, MN USA) (Minneapolis, USA). The B16 murine melanoma was a kind gift from Dr. Sinisa Radulovic (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia). Human melanoma A375 and colon cancer HCT116 were a kind gift from Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Human lung carcinoma A549, breast adenocarcinoma MCF7 and normal lung fibroblast cell line MRC5 were purchased from ATCC. Cells are routinely kept 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. After standard trypsinization, cells were seeded at 1×10^4/well in 96-well plates for viability determination and 2.5×10^5/well in 6-well plate for flow cytometry.

2.2.2. Determination of cell viability by crystal violet assay

The viability of adherent cells was evaluated with crystal violet (CV) assay [26,27]. Complexes were dissolved in DMSO (stock solutions 200 mM) and ligands in absolute ethanol (stock solutions 18 mM) and then serial dilutions were made in culture medium. Cells were treated with the wide range of doses of the drugs for 24 h and then cells were fixed with methanol 10 min at RT. In parallel, control cells were exposed to the same amount of solvents corresponding to their content in the solutions of complexes or ligands. After fixation cells were stained with 1% crystal violet solution for 15 min, washed, dried and the dye was dissolved in 33% acetic acid. The absorbance of dissolved dye was measured at 540 nm with the reference wavelength at 640 nm. Results are presented as percentage of control that was arbitrarily set to 100%.

2.2.3. Cell cycle analysis

Cells were treated with IC_{50} dose of **C1** or **C2** for 24 h, then trypsinized and fixed in 70% ethanol at 4 °C overnight. After washing in PBS, cells were stained with PI (20 μg/ml) and RNase (0.1 mg/ml) for 30 min at 37 °C in the dark. Red fluorescence was analyzed with FACS Calibur flow cytometer (BD, Heidelberg, Germany). The distribution of cells in different cell cycle phases was determined with Cell Quest Pro software (BD) [26,28].

2.2.4. AnnexinV-FITC/PI staining and caspase detection

Cells were treated with IC_{50} dose of **C1** or **C2** for 24 h, then trypsinized and stained with AnnV-FITC/PI (Biotium, Hayward, CA) or apostat according to the manufacturer's instruction. Cells were analyzed with FACS Calibur flow cytometer (BD, Heidelberg, Germany) using Cell Quest Pro software (BD) [29,30].

2.2.5. PI staining on chamber slide

Cells were treated with IC_{50} dose of **C1** or **C2** for 24 h, and then fixed in 4% PFA 15 min at room temperature. Cells were stained 2 min with 20 μg/ml PI covered with fluorescent mounting media (Dako, Glostrup, Denmark) and analyzed under the fluorescent microscope. Between all steps cells were washed 2–3 times with PBS [30,31].

2.2.6. Statistical analysis

The results are presented as mean ± SD of triplicate observations from the representative of three experiments, except if indicated otherwise. The significance of the difference between treatments and control was analyzed by ANOVA followed by Student–Newman–Keuls test. $P < 0.05$ was considered significant.

3. Results

3.1. Synthesis of the ligands

3.1.1. Synthesis of O,O′-diisobuthyl-(S,S)-ethylenediamine-N,N′-di-2-(3-cyclohexyl)propanoate dihydrochloride, *L1*·2HCl

Into the ice-cooled absolute isobutanol (15 mL) the thionyl chloride (2.00 mL) and (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid dihydrochloride (1.20 g, 2.70 mmol) were added. After 1 h of stirring at 0 °C, suspension was refluxed for 16 h. The resulting mixture was filtered and left to crystallize. The white product was filtered off, washed with isobutanol and dried in *vacuo*. Compound **L1** is a white powder. Yield: 810 mg (53.79%); Mp: 220 °C; ^{1}H NMR (500.26 MHz, TFA-d_1): δ = 1.19–1.27 (m, $(CH_3)_2CHCH_2-$, 12H; C5, 4H), 1.38–1.52 (m, C6, 4H; C7, 2H), 1.77 (m, C4, 2H), 1.88–2.05 (m, C5,6, 8H; C7, 2H), 2.19 (m, $-CH_2Cy$, 4H; $(CH_3)_2CHCH_2-$, 2H), 4.12 and 4.18 ($-NH_2CH_2CH_2NH_2-$, 4H), 4.32 and 4.37 (m, $-CH_2OOC-$, 4H), 4.53 ppm (t, $-OOCCHNH_2-$, 2H); ^{13}C NMR (125.79 MHz, TFA-d_1): 19.58 ($(CH_3)_2CHCH_2-$), 27.51 and 27.63

(*C*7, *C*6), 29.71 (−*C*H(CH_3)$_2$), 35.0 (*C*4), 36.37 (*C*5), 39.69 (−*C*H_2Cy), 46.66 (−$NH_2CH_2CH_2NH_2$−), 62.81 (−OOC*C*HNH_2−), 77.25 (−*C*H_2OOC−), 172.92 ppm (*C*1); IR (ATR): $\tilde{\nu}$ = 2928.8, 2844.6, 2592.0, 2529.9, 2415.4, 2356.4, 2216.6, 1742.7, 1540.6, 1471.9, 1452.8, 1219.4, 1180.2, 1066.5, 999.1, 807.1 cm^{-1}; MS (LC-ESI): *m*/*z* (%): 481.40 (26.83) $[M - 2HCl + H]^+$; Anal. Calcd. for $C_{28}H_{54}Cl_2N_2O_4$, %: C 60.76, H 9.76, N 5.06. Found, %: C 60.29, H 9.87, N 4.81.

3.1.2. Synthesis of *O*,*O*′-diisoamyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dihydrochloride, **L2**·2HCl

O,*O*′-diisoamyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoate dihydrochloride, was prepared as described above, but using absolute isoamyl alcohol, instead of isobutanol. Compound **L2** is a white powder. Yield: 890 mg (56.34%); Mp: 224 °C; ^{1}H NMR (500.26 MHz, TFA-d_1): δ = 1.16–1.28 (m, (CH_3)$_2$CHCH_2CH_2–, 12H; C5, 4H; (CH_3)$_2$C*H*CH_2CH_2–, 2H), 1.39–1.50 (m, C6, 4H; C7, 2H), 1.76–2.05 (m, C4, 2H; C5, C6, 8H; C7, 2H; ((CH_3)$_2$CHCH_2CH_2–), 4H); 2.18 (m, −CH_2Cy, 4H); 4.14 and 4.19 (m, −$NH_2CH_2CH_2NH_2$−, 4H), 4.53 and 4.58 (m, −CH_2OOC−, 4H), 4.67 ppm (m, −OOCC*H*NH_2−, 2H); ^{13}C NMR (125.79 MHz, TFA-d_1): 11.76 and 17.02 ((CH_3)$_2$CHCH_2CH_2–), 22.92 ((CH_3)$_2$CHCH_2CH_2–), 27.24 and 27.65 (*C*7, *C*6), 35.00 (*C*4), 36.30 (*C*5), 38.83 ((CH_3)$_2$CHCH_2CH_2–), 39.62 (−*C*H_2Cy), 46.63 (−$NH_2CH_2CH_2NH_2$−), 62.84 (−OOCCHNH_2−), 70.0 (−*C*H_2OOC−), 172.84 ppm (*C*1); IR (ATR): $\tilde{\nu}$ = 2962.4, 2928.2, 2849.9, 2704.9, 2611.6, 2528.5, 2470.1, 2408.6, 2220.1, 1746.4, 1539.3, 1481.4, 1455.5, 1320.5, 1259.4, 1211.3, 1176.5, 1072.7, 998.1, 947.7, 805 cm^{-1}; MS (LC-ESI): *m*/*z* (%): 509.43 (6.82) $[M - 2HCl + H]^+$; Anal. Calcd. for $C_{30}H_{58}Cl_2N_2O_4$, %: C 61.94, H 10.05, N 4.82. Found, %: C 61.54, H 10.21, N 4.79.

3.2. Synthesis of organometallic complexes

3.2.1. Synthesis of [(η^6-*p*-cymene)Ru(L1)Cl], **C1**

A suspension of ligand *O*,*O*′-diisobutyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dihydrochloride (0.11 g, 0.20 mmol) in methanol (5 mL) priorly neutralized with the lithium hydroxide monohydrate (16.78 mg, 0.40 mmol), was added in the mixture of methanol/chloroform (10 mL, 1:1) solution of [(η^6-*p*-cymene)$RuCl_2$]$_2$ (61.23 mg, 0.10 mmol). The reaction mixture was stirred at 40 °C for 3 h. The orange solution was concentrated under reduced pressure to *ca.* 10 mL and solid NH_4PF_6 (57.05 mg, 0.35 mmol) was added. The mixture was stirred at room temperature for 1 h and a yellow–orange product was precipitated. This was filtered off, washed with methanol and dried in *vacuo*. Yield: 92 mg (51.31%). Crystals suitable for X-ray crystallographic study were obtained from mother liquor at 4 °C. Mp: 182 °C; ^{1}H NMR (500.26 MHz, DMSO-d_6): δ = 0.90 and 0.98 (d, (CH_3)$_2$CHCH_2O–, 12H); 1.10 (m, C5, C6, 8H; C7, 2H), 1.24 (dd, (CH_3)$_2$CH(arene), 6H)), 1.55–1.96 and 2.18 (m, C4, 2H; C5, C6, 8H; C7, 2H; −CH_2Cy, 4H; −NHCH_2CH_2NH–, 4H), 2.25 (s, CH_3(arene), 3H), 2.57 (m, (CH_3)$_2$C*H*CH_2O–, 2H), 2.82 (sept, −C*H*(CH_3)$_2$, 1H), 3.91 and 4.03 (m, d (CH_3)$_2$CHCH_2OOC–, 4H), 3.65 and 4.17 (td, −OOCC*H*NH–, 2H), 5.08 (d, C12*H*(arene), 1H), 5.36 (d, C13*H*(arene), 1H), 5.81 (d, C12′*H*(arene), 1H), 6.05 (d, C13′*H*(arene), 1H), 6.82 and 7.05 ppm (td, m, −CH_2N*H*N*H*CH_2–, 2H); ^{13}C NMR (125.79 MHz, DMSO-d_6): δ = 16.04 (*C*H_3(arene)), 18.79 ((CH_3)$_2$CHCH_2O–), 21.52 (−CH(CH_3)$_2$), 25.31 (*C*7), 25.74 (*C*6), 27.05 ((CH_3)$_2$CHCH_2O–), 30.38 (−*C*H(CH_3)$_2$), 31.36 (*C*5), 34.08 (*C*4), 37.89 (−*C*H_2Cy), 45.96 and 50.12 (−NHCH_2CH_2NH–), 58.82 and 60.33 (−OOC*C*HNH–), 70.90 (CH_3)$_2$CHCH_2OOC, 74.36 and 75.41 (*C*12H(arene), *C*12′H(arene)), 85.69 and 86.35 (*C*13H(arene), *C*13′H(arene)), 92.29 (*C*14(arene)), 111.82 (*C*11(arene)), 171.85 and 173.12 ppm ((CH_3)$_2$CHCH_2OO*C*–); IR (ATR): $\tilde{\nu}$ = 3253.9, 2926.8, 2852.8, 1730.7, 1469.9, 1450.0, 1383.7, 1189.4, 839.6 cm^{-1}; UV/Vis (CH_3CN): λ_{max} (ε) = 325.96 (107), 411.06 nm (67 L mol^{-1} cm^{-1}); MS (LC-ESI): *m*/*z* (%): 751.38 (100) $[M]^+$, 752.38 (50.31) $[M + H]^+$; Anal. Calcd. for $C_{38}H_{66}ClF_6N_2O_4PRu$, %: C 50.91, H 7.42, N 3.13. Found, %: C 50.85, H 7.21, N 3.17.

3.2.2. Synthesis of [(η^6-*p*-cymene)Ru(L2)Cl], **C2**

A suspension of ligand *O*,*O*′-diisoamyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dihydrochloride (0.13 g, 0.22 mmol) in methanol (5 mL) priorly neutralized with the lithium hydroxide monohydrate (18.46 mg, 0.44 mmol), was added to the mixture of methanol/chloroform (10 mL, 1:1) solution of [(η^6-*p*-cymene)$RuCl_2$]$_2$ (67.35 mg, 0.11 mmol). The reaction mixture was stirred at 40 °C for 3 h. Then the orange solution was concentrated under reduced pressure to *ca.* 10 mL. After addition of NH_4PF_6 (62.75 mg, 0.38 mmol) the fine yellow-orange solid was isolated as described for complex **C1**. Yield: 114 mg (62.85%); Mp: 184 °C; ^{1}H NMR (500.26 MHz, DMSO-d_6): δ = 0.78–1.38 (dt, (CH_3)$_2$CHCH_2 CH_2OOC–, 12H; m, C5, C6, 8H; C7, 2H; (CH_3)$_2$CHCH_2CH_2OOC–, 4H; dd, (CH_3)$_2$CH(arene), 6H), 1.54–1.95 and 2.19 (m, C4, 2H; C5, C6, 8H; C7, 2H; −CH_2Cy, 4H; −NHCH_2CH_2NH–, 4H), 2.25 (s, CH_3(arene), 3H), 2.56 (m, (CH_3)$_2$C*H*CH_2OOC–, 2H), 2.81 (sept, −C*H*(CH_3)$_2$, 1H), 3.64 and 4.07 (m, −OOCC*H*NH–, 2H), 4.18 and 4.29 (m, (CH_3)$_2$CHCH_2CH_2OOC–, 4H), 5.05 (d, C12*H*(arene), 1H), 5.35 (d, C13*H*(arene), 1H), 5.80 (d, C12′*H*(arene), 1H), 6.07 (d, C13′*H*(arene), 1H), 6.83 and 7.03 ppm (m, −CH_2N*H*N*H*CH_2–, 2H); ^{13}C NMR (125.79 MHz, DMSO-d_6): δ = 13.83 and 20.81 ((CH_3)$_2$CHCH_2CH_2 OOC–), 16.01 (*C*H_3(arene)), 21.77 (−CH(*C*H_3)$_2$), 22.05 ((CH_3)$_2$C*H*CH_2 CH_2OOC–), 25.37 (*C*7), 25.77 (*C*6), 27.66 ((CH_3)$_2$CHCH_2CH_2OOC–), 30.46 (−*C*H(CH_3)$_2$), 31.39 (*C*4), 34.27 (*C*5), 37.91 (−*C*H_2Cy), 45.91 and 50.13 (−NHCH_2CH_2NH–), 58.83 and 60.39 (−OOC*C*HNH–), 64.75 and 65.01 ((CH_3)$_2$CHCH_2CH_2OOC–), 74.15 and 75.28 (*C*12H(arene), *C*12′H(arene)), 86.16 and 86.78 (*C*13H(arene), *C*13′H(arene)), 92.13 (*C*14(arene)), 112.05 (*C*11(arene)), 171.88 and 173.01 ppm ((CH_3)$_2$CHCH_2CH_2OO*C*–); IR (ATR): $\tilde{\nu}$ = 3433.7, 3261.0, 2927.7, 2853.8, 1732.9, 1451.4, 1349.0, 1213.2, 1189.0, 1040.0, 987.5, 961.3, 843.9, 558.6 cm^{-1}; UV/Vis (CH_3CN): λ_{max} (ε) = 333.62 (86), 412.77 nm (58 L mol^{-1} cm^{-1}); MS (LC-ESI): *m*/*z* (%): 779.41 (100) $[M]^+$, 780.41 (50.80) $[M + H]^+$; Anal. Calcd. for $C_{40}H_{70}ClF_6N_2O_4PRu$, %: C 51.97, H 7.63, N 3.03. Found, %: C 51.81, H 7.41, N 2.98.

Table 1
Crystal data and details of data collection for [(η^6-*p*-cymene)Ru(L1)Cl][PF_6] (**C1**).

Empirical formula	$C_{38}H_{66}ClF_6N_2O_4PRu$
Fw	896.42
Space group	*P*1
a [Å]	10.2886(5)
b [Å]	14.9911(7)
c [Å]	15.7340(7)
α [°]	63.685(2)
β [°]	83.782(3)
γ [°]	76.489(3)
V [Å^3]	2115.05(17)
Z	2
λ [Å]	0.71073
ρ_{calcd} [g cm^{-3}]	1.408
Crystal size [mm^3]	0.12 × 0.08 × 0.04
T [K]	100(2)
μ [mm^{-1}]	0.538
R_1[a]	0.0509
wR_2[b]	0.1211
GOF[c]	1.050

[a] $R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$.

[b] $wR_2 = \{\sum[(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)^2]\}^{1/2}$.

[c] GOF = $\{\sum[(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$, where *n* is the number of reflections and *p* is the total number of parameters refined.

3.3. X-ray diffraction

X-ray diffraction measurements were performed on a Bruker X8 APEXII CCD diffractometer. Single crystal was positioned at 40 mm from the detector, and 1935 frames were measured, each for 40 s over 1° scan width. The data were processed using SAINT software [32] Crystal data, data collection parameters, and structure refinement details are given in Table 1. The structure was solved by direct methods and refined by full-matrix least-squares techniques. Nonhydrogen atoms were refined with anisotropic displacement parameters, except those involved in the observed disorder (vide infra). Hydrogen atoms were inserted in calculated positions and refined with a riding model. Two cyclohexane rings and one ester function in the second complex cation were found to be disordered over two positions with s.o.f. 0.6:0.4, 0.5:0.5 and 0.5:0.5, respectively. The disorder was resolved by using SADI and EADP tools implemented in *SHELXL*. The following computer programs and hardware were used: structure solution, *SHELXS-97* and refinement, *SHELXL-97* [33]; molecular diagrams, ORTEP [34] computer, Intel CoreDuo.

4. Discussion

4.1. Synthesis of ligands and complexes

Isobutyl and isoamyl ester of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (Fig. 1) have been obtained by the reaction of acid, corresponding absolute alcohol and thionyl chloride under reflux. The compounds are soluble in trifluoroacetic acid, absolute ethanol and poorly soluble in DMSO.

By exploring the μ-chlorido-bridge splitting reaction of $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ with L1·2HCl and/or L2·2HCl in methanol/chloroform at 40 °C, with subsequent addition of ammonium hexafluorophosphate, complexes $[(\eta^6\text{-}p\text{-cymene})Ru(L1)Cl]$ (**C1**) and $[(\eta^6\text{-}p\text{-cymene})Ru(L2)Cl]$ (**C2**) (Fig. 2) have been prepared in good yields. The complexes are soluble in DMSO and insoluble in water.

4.2. Spectroscopic studies

Characteristic absorption bands for aliphatic esters and C=O stretching vibrations were found in the IR spectra of the ligands (1743 and 1746 cm^{-1}) (Fig. S1) and complexes (1731 and 1733 cm^{-1}). Absorption bands due to C=O stretching vibrations in the complexes show the same wave number as in the free ligands, indicating non-involvement of those groups into coordination. The

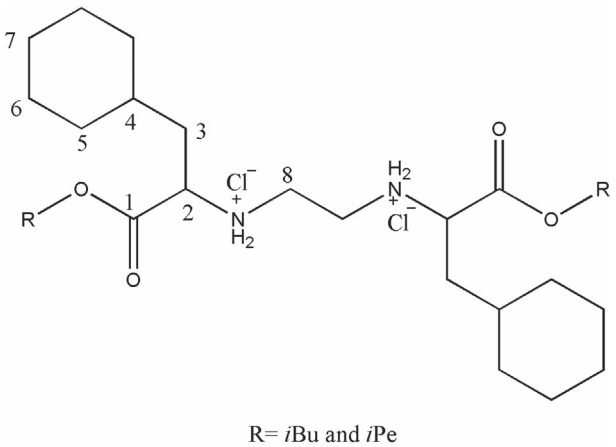

Fig. 1. Synthesized ligands **L1** and **L2**.

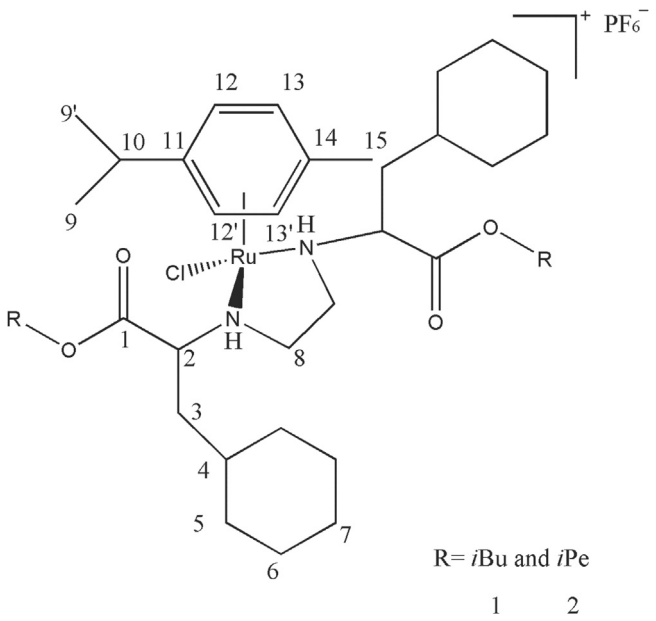

Fig. 2. Ru(II) complexes **C1** and **C2**.

IR spectra of complexes show characteristic absorption bands for *val*(N–H) vibrations at 3254 and 3261 cm^{-1}. The ^1H NMR spectra of complexes showed characteristic resonances of η^6-*p*-cymene and the diamine moiety. Upon complexation to the metal, the symmetry of the ligand environment is lowered. In the spectra of complexes this is manifested by the non equivalence of the chemically equivalent protons in free ligands, –(ROOC)C**H**–N**H**–CH_2–CH_2–N**H**–C**H**(COOR)–. This is also observed in the cymene ring protons which appear as four doublets, in contrast to the one signal seen in the $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$. In both cases this is induced by lower symmetry of the coordination environment. Signals of –**H**N–C**H**$_2$–C**H**$_2$–N**H**– in complexes are shifted to lower

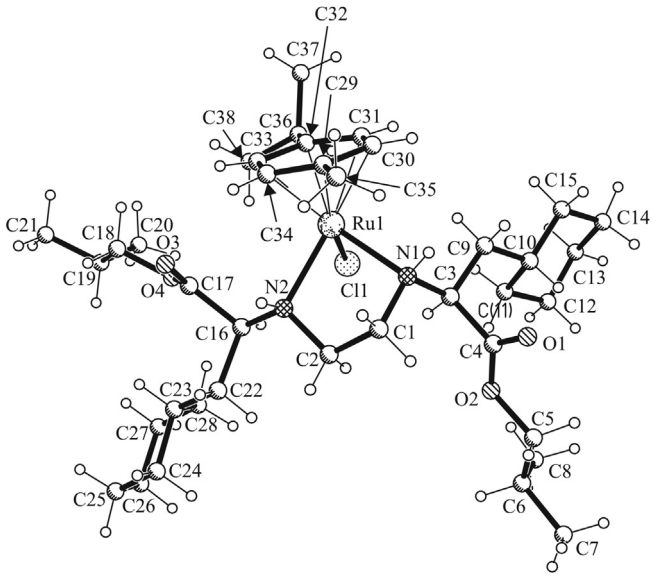

Fig. 3. A view of one crystallographically independent complex cation $[(\eta^6\text{-}p\text{-cymene})Ru(L1)Cl]^+$ of **C1**. Selected bond distances (Å), bond angles and torsion angles (°): Ru1–Cl1 2.3888(18), Ru1–N1 2.209(5), Ru1–N2 2.206(5), Ru1–C29 2.203(7), Ru1–C30 2.188(6), Ru1–C31 2.169(7), Ru1–C32 2.224(7), Ru1–C33 2.211(6), Ru1–C34 2.155(7); N1–Ru1–N2 81.26(18); $\Theta_{N1-C1-C2-N2} = -59.5(7)$.

Table 2
IC_{50} [μM] values of C1 and C2 after 24 h of action against selected tumor cell lines. IC_{50} values were calculated as mean ± SD from three independent experiments.

Compound	B16	A375	IC_{50} (μM) HCT116	A549	MCF7	MRC5
C1	5.5 ± 0.9	2.9 ± 1.1	4.4 ± 0.4	6.4 ± 2.5	5.0 ± 0.1	8.7 ± 0.4
C2	7.4 ± 3.2	3.5 ± 1.6	5.5 ± 0.9	8.0 ± 0.3	7.8 ± 0.6	12.0 ± 3.4

chemical shifts, due to coordination to metal. Characteristic septet at 2.81 ppm is assigned to one proton from isopropyl group of aromatic ligand. In the ^{1}H NMR spectra of ligands (recorded in TFA, Fig. S2), NH protons could not be detected, because of the fast exchange of these protons with solvent protons. In the ^{1}H NMR spectra of ligands (recorded in DMSO), NH protons were seen at 9.8 ppm. A lowered symmetry of the coordination environment was also evidenced by the ^{13}C NMR spectra, particularly for –(ROOC)**C**H–NH–**C**H_2–**C**H_2–NH–**C**H(COOR)–, the cymene ring carbons and carbonyl groups. The cymene carbons appear as six signals, unlike four signals in starting ruthenium cymene complex. Signals of –HN–**C**H_2–**C**H_2–NH– in complexes are downfield shifted in comparison with free ligand.

The mass spectra of the ligands contain peaks assigned to the $[M - 2HCl + H]^+$ ion, which is in agreement with their calculated molecular mass. Parent ions of complexes were detectable *m/z* at 751 for **C1** and *m/z* 779 (Fig. S3) for **C2** and they are in accordance with expected values.

4.3. X-ray studies

The result of X-ray diffraction study of complex **C1** is shown in Fig. 3 with selected bond distances and bond angles given in the caption. The complex crystallized in the triclinic non-centrosymmetric space group *P*1 with two complex cations $[(\eta^6$-*p*-cymene)Ru(L1)Cl$]^+$ and two counteranions $[PF_6]^-$ in the unit cell. Each ruthenium atom adopts a typical "piano-stool" configuration with one chlorido ligand and two nitrogen donors of the organic ligand as the three legs. Both sp^3 hybridized nitrogen atoms are chiral with the same chirality *S*. Upon binding of the bidentate ligand to ruthenium a five-membered chelate ring is formed. A twist conformation of this metallocycle is adopted in the complex cation shown in Fig. 3, while an envelope one in the second cation.

4.4. Anticancer activity

To explore the anticancer potential of both ligands, **L1** and **L2** and complexes **C1** and **C2** several cell lines with different origin and well defined cell specificity were selected. Cells were exposed to a wide range of tested drug doses and cell viability was estimated after 24 h incubation time. In parallel, cells were treated with ligands **L1** and **L2** in the same dose range. Results clearly indicate that both complexes possess a strong anticancer potential *in vitro* (Table 2). Determined IC_{50} doses were even lower than previously found upon the treatment of different adherent cell lines with ruthenium–cymene complexes with cyclohexyl-functionalized ethylendiamine-*N*,*N*′-diacetate-type ligands or cisplatin [35]. On the other hand, highest dose of the ligand compounds showed only slight decrease of the cell viability (10–40%) (Table 3).

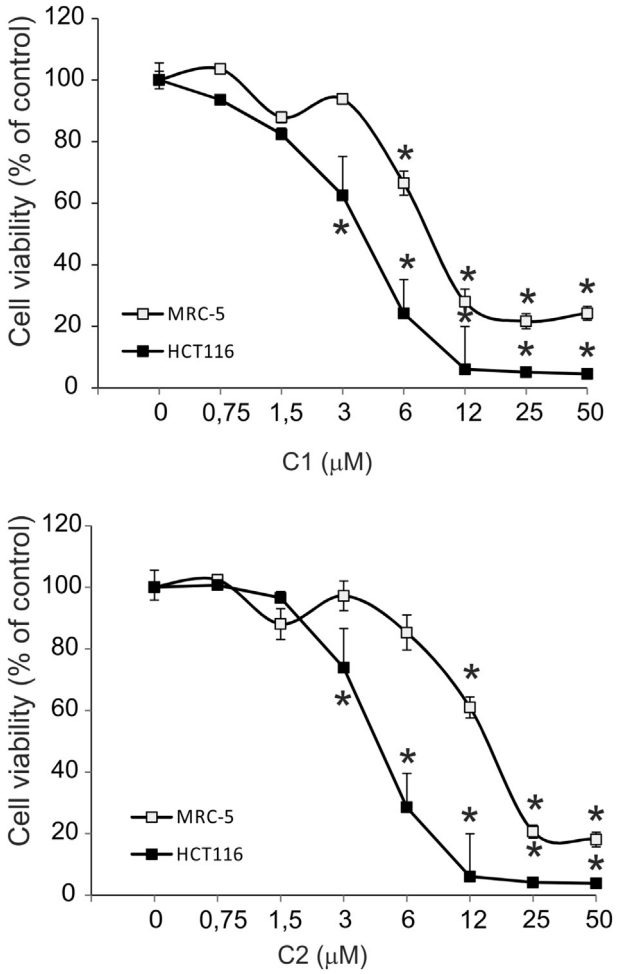

Fig. 4. The effect of **C1** and **C2** on viability of normal and colon cancer cells. MRC-5 fibroblasts and HCT116 colon cancer cells (1×10^4) were treated with different doses of **C1** and **C2** for 24 h, after which cell viability was measured by CV assay. The data are presented as mean ± SD from representative of three independent experiments.

Given that the new synthesized complexes differ only in the ester part, compared to the previously synthesized complexes of this type, a very good biological activity of these complexes was expected. In parallel, primary fibroblasts-MRC-5 were remarkably less affected by these compounds, revealing selective potential of novel drugs against malignant cells (Fig. 4). Importantly, colon cancer cells HCT116 ($IC_{50} > 120$ μM) resistant to one of the most potent anticancer drug cisplatin were highly sensitive to mentioned compounds [30]. Low IC_{50} doses as well as the ability to overcome cisplatin developed resistance present one of the greatest advantages of newly created compounds in comparison to commercially used metal based drugs in oncotherapy [36].

4.5. Cell cycle arrest and apoptosis

Cisplatin resistant cells HCT116 were selected for further experiments. To elucidate the mechanism involved in antitumor

Table 3
Viability of cell lines upon the treatment with 50 μM L1 and L2. Values are expresses as % of control and calculated as mean ± SD from three independent experiments.

Compound	B16	A375	HCT116	A549	MCF7	MRC5
L1	101.0 ± 1.2	97.3 ± 1.5	96.5 ± 1.8	100.5 ± 1.9	102.7 ± 4.8	93.4 ± 2.1
L2	71.5 ± 4.1	78.9 ± 0.3	81.9 ± 1.0	84.7 ± 3.15	60.6 ± 3.1	52.0 ± 4.1

activity of **C1** and **C2**, cells were exposed to IC_{50} concentrations of each compound and cell cycle distribution was determined after 24 and 48 h. Remarkable arrest of cells in G0/G1 phase accompanied by an increased percentage of cells in subG phase was observed after first 24 h (Fig. 5). In addition, progressive accumulation of cells in G2/M phase as well as hypodiploid cells for approximately 20% was evident in cultures exposed to **C1** or **C2** after additional 24 h of incubation (data not shown). Decreased number of viable cells as well as apoptotic nuclei was visualized by PI staining at this time point (Fig. 5, right panel).

One of the first signs of apoptosis is externalization of phosphatidylserine residues [37]. At the intracellular level, death signal will be delivered most often with caspase activation, but in some cases it will be completed even without functional caspases [38]. To confirm the hypothesis that novel drugs triggered classic apoptotic process, cells were exposed to **C1** or **C2** and after 24 h Ann/PI double

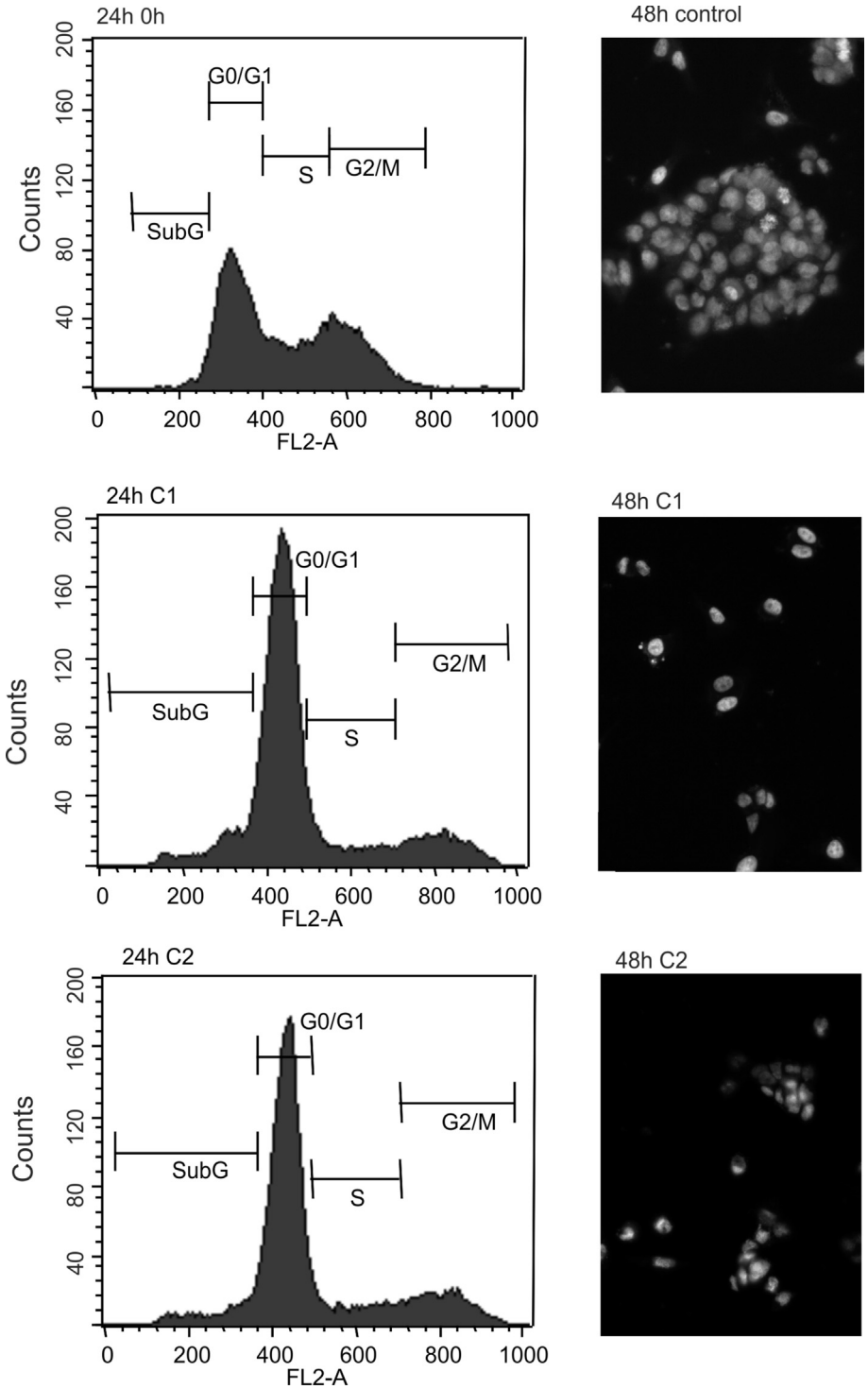

Fig. 5. The effect of **C1** and **C2** on cell cycle distribution. HCT116 cells (2×10^5/well) were exposed to IC_{50} doses of **C1** and **C2** for 24 h and cell cycle distribution (left panel) was evaluated. For determination of nuclear morphology cells were stain with PI and analyzed by fluorescent microscopy (right panel).

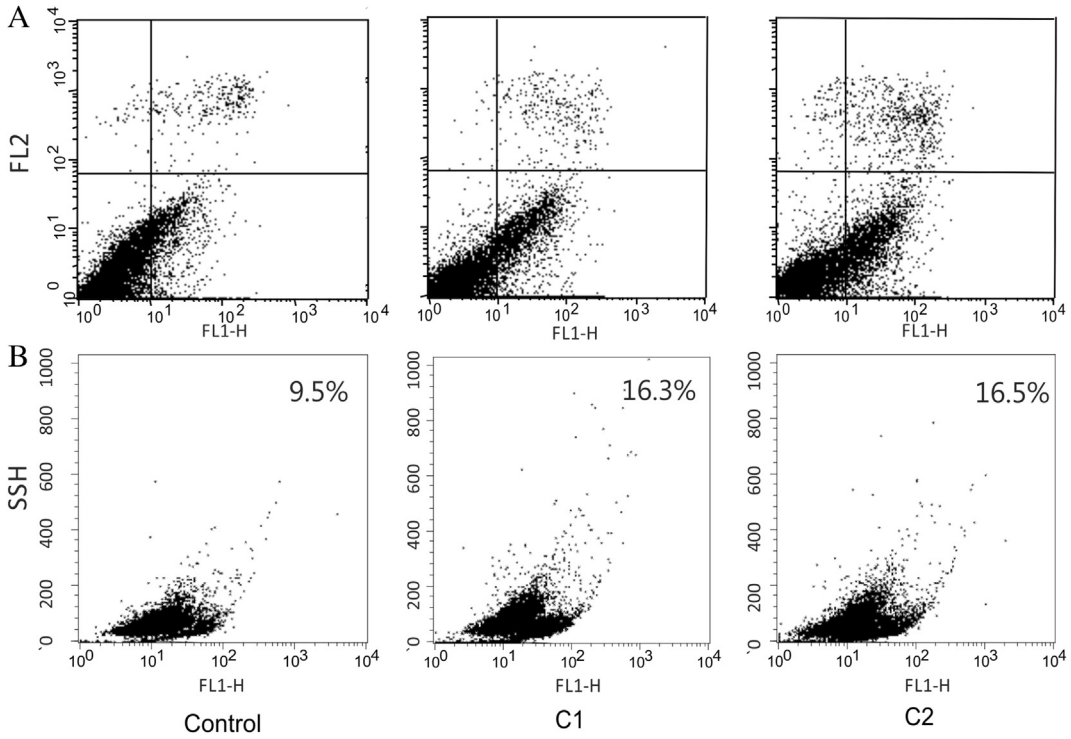

Fig. 6. The effect of **C1** and **C2** on apoptotic process. HCT116 cells (2×10^5/well) were exposed to IC_{50} doses of **C1** and **C2** and Ann/PI double staining (A) and ApoStat staining (B) after 24 h were done.

staining was performed. As displayed in Fig. 6 (upper panel), it is evident that tested compounds promoted strong apoptotic process with remarkable accumulation of cells with early apoptotic profile (Ann^+PI^-) as well as double positive, necrotic cells. In parallel, apostat staining revealed distinct population of cells with elevated caspase activity upon the treatment with **C1** and **C2** for 24 h (Fig. 6, lower panel).

The obtained data are in accord with our previous results on ruthenium–cymene complexes with cyclohexyl-functionalized ethylendiamine-*N*,*N*′-diacetate-type ligands [35]. In brief, Ru–cymene complex with butyl ester as ligand, induced typical apoptosis with both phosphatidyl internalization and DNA fragmentation accompanied by caspase activation in HL-60 cells confirming that induction of apoptotic process is a unique feature of these classes of metalo-drugs. However, in the majority of cells caspase activity was not changed. In line with this, treatment of **C1** or **C2** with caspase inhibitor ZVAD restores the viability of approximately 8%. Taken together, apoptosis triggered by both tested compounds is just partly regulated by caspases. This implicated the advantage of novel compounds to promote death even independently of caspase activation. In addition, this type of caspase independent apoptosis eliminated undesirable effects of caspase activation, such as signal for compensatory proliferation [39].

5. Conclusion

The ruthenium(II) arene complexes containing new alkyl esters of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid as chelating ligand have been synthesized. Ligands and complexes were characterized by NMR, IR spectroscopy and ESI-MS spectrometry and the structure of C1 was determined by X-ray diffraction. The cytotoxic studies showed that the synthesized complexes exhibited strong biological activity. IC_{50} values of the tested complexes were even 10–30 times lower than those of the ligand counterparts or even cisplatin under same experimental conditions. It is noteworthy, that the investigated complexes are particularly active towards cisplatin resistant HCT116 cell line. Cell cycle arrest and apoptosis are responsible for cytotoxicity of these complexes. Of particular interest and importance is the examination of the anticancer activity of these compounds *in vivo*, which will be the subject of future research.

Acknowledgments

This work was supported by the Ministry of Education, Science and Technological development of the Republic of Serbia, grant numbers 172035 and 173013.

Appendix A. Supplementary material

CCDC 945341contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Appendix B. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jorganchem.2013.08.041.

References

[1] A.G. Quiroga, J. Inorg. Biochem. 114 (2012) 106–112.
[2] G.P. Stathopoulos, D. Antoniou, J. Dimitroulis, J. Stathopoulos, K. Marosis, P. Michalopoulou, Cancer Chemother. Pharmacol. 68 (2011) 945–950.
[3] G. Sava, G. Jaouen, E.A. Hillard, A. Bergamo, Dalton Trans. 41 (2012) 8226–8234.
[4] H.P. Varbanov, M.A. Jakupec, A. Roller, F. Jensen, M. Galanski, B.K. Keppler, J. Med. Chem. 56 (2013) 330–344.
[5] M. Groessl, C.G. Hartinger, Anal. Bioanal. Chem. 405 (2013) 1791–1808.

[6] F. Giannini, J. Furrer, G. Süss-Fink, C.M. Clavel, P.J. Dyson, J. Organomet. Chem. 741 (2013) 41–48.
[7] W.H. Ang, A. Casini, G. Sava, P.J. Dyson, J. Organomet. Chem. 696 (2011) 989–998.
[8] R. Gogna, E. Madan, B.K. Keppler, U. Pati, Br. J. Pharmacol. 166 (2012) 617–636.
[9] A.M. Pizzaro, A. Habtemariam, P.J. Sadler, Organomet. Chem. 32 (2010) 21–56.
[10] S. Grgurić-Šipka, I. Ivanović, G. Rakić, N. Todorović, N. Gligorijević, S. Radulović, V.B. Arion, B.K. Keppler, Ž.Lj. Tešić, Eur. J. Med. Chem. 45 (2010) 1051–1058.
[11] M.V. Babak, S.M. Meier, A.A. Legin, M.S. Adib Razavi, A. Roller, M.A. Jakupec, B.K. Keppler, C.G. Hartinger, Chem. Eur. J. 19 (2013) 4308–4318.
[12] W. Kandioller, A. Kurzwernhart, M. Hanif, S.M. Meier, H. Henke, B.K. Keppler, C.G. Hartinger, J. Organomet. Chem. 696 (2011) 999–1010.
[13] J.H. Kasser, W. Kandioller, C.G. Hartinger, A.A. Nazarov, V.B. Arion, P.J. Dyson, B.K. Keppler, J. Organomet. Chem. 695 (2010) 875–881.
[14] F.A. Khan, B. Therrien, G. Süss-Fink, O. Zava, P.J. Dyson, J. Organomet. Chem. 730 (2013) 49–56.
[15] J. Kljun, A.K. Bytzek, W. Kandioller, C. Bartel, M.A. Jakupec, C.G. Hartinger, B.K. Keppler, I. Turel, Organometallics 30 (2011) 2506–2512.
[16] M.A. Jakupec, M. Galanski, V.B. Arion, C.G. Hartinger, B.K. Keppler, Dalton Trans. (2008) 183–194.
[17] P.J. Dyson, G. Sava, Dalton Trans. (2006) 1929–1933.
[18] H. Chen, J.A. Parkinson, S. Parsons, R.A. Coxall, R.O. Gould, P.J. Sadler, J. Am. Chem. Soc. 124 (2002) 3064–3082.
[19] H. Chen, J.A. Parkinson, R.E. Morris, P.J. Sadler, J. Am. Chem. Soc. 125 (2003) 173–186.
[20] R. Pettinari, C. Pettinari, F. Marchetti, C.M. Clavel, R. Scopelliti, P.J. Dyson, Organometallics 32 (2013) 309–316.
[21] A.R. Timerbaev, C.G. Hartinger, S.S. Aleksenko, B.K. Keppler, Chem. Rev. 106 (2006) 2224–2248.
[22] K.S. Lovejoy, S.J. Lippard, Dalton Trans. (2009) 10651–10659.
[23] J.M. Lazić, Lj. Vučićević, S. Grgurić-Šipka, K. Janjetović, G.N. Kaludjerović, M. Misirkić, M. Gruden-Pavlović, D. Popadić, R. Paschke, V. Trajković, T.J. Sabo, Chem. Med. Chem. 5 (2010) 881–889.
[24] S. Misirlic Dencic, J. Poljarevic, U. Vilimanovich, A. Bogdanovic, A.J. Isakovic, T. Kravic Stevovic, M. Dulovic, N. Zogovic, A.M. Isakovic, S. Grguric-Sipka, V. Bumbasirevic, T. Sabo, V. Trajkovic, I. Markovic, Chem. Res. Toxicol. 25 (2012) 931–939.
[25] S.B. Jensen, S.J. Rodger, M.D. Spicer, J. Organomet. Chem. 556 (1998) 151–158.
[26] L.E. Mihajlović, A. Savić, J. Poljarević, I. Vučković, M. Mojić, M. Bulatović, D. Maksimović-Ivanić, S. Mijatović, G.N. Kaluđerović, S. Stošić-Grujičić, Đ. Miljković, S. Grgurić-Šipka, T.J. Sabo, J. Inorg. Biochem. 109 (2012) 40–48.
[27] S. Mijatovic, D. Maksimovic-Ivanic, J. Radovic, D. Popadic, M. Momcilovic, L. Harhaji, D. Miljkovic, V. Trajkovic, Cell. Mol. Life Sci. 61 (2004) 1805–1815.
[28] S. Mijatovic, D. Maksimovic-Ivanic, J. Radovic, Dj. Miljkovic, Lj. Harhaji, O. Vuckovic, S. Stosic-Grujicic, M. Mostarica Stojkovic, V. Trajkovic, Cell. Mol. Life Sci. 62 (2005) 589–598.
[29] S. Mijatovic, D. Maksimovic-Ivanic, J. Radovic, D. Miljkovic, G.N. Kaludjerovic, T.J. Sabo, V. Trajkovic, Cell. Mol. Life Sci. 62 (2005) 1275–1282.
[30] G.N. Kaluđerović, S.A. Mijatović, B.B. Zmejkovski, M.Z. Bulatović, S. Gómez-Ruiz, M.K. Mojić, D. Steinborn, D.M. Miljković, H. Schmidt, S.D. Stošić-Grujičić, T.J. Sabo, D.D. Maksimović-Ivanić, Metallomics 4 (2012) 979–987.
[31] D. Maksimovic-Ivanic, S. Mijatovic, L. Harhaji, D. Miljkovic, D. Dabideen, K. Fan Cheng, K. Mangano, G. Malaponte, Y. Al-Abed, M. Libra, G. Garotta, F. Nicoletti, S. Stosic-Grujicic, Mol. Cancer Ther. 7 (2008) 510–520.
[32] SAINT-plus, Version 7.06a and APEX2, Bruker-Nonius AXS Inc., Madison, WI, 2004.
[33] G.M. Sheldrick, Acta Crystallogr. A64 (2008) 112–122.
[34] M.N. Burnett, G.K. Johnson, ORTEPIII. Report ORNL-6895, OAK Ridge National Laboratory, Tennessee, USA, 1996.
[35] A. Savić, M. Dulović, J.M. Poljarević, S. Misirlić-Denčić, M. Jovanović, A. Bogdanović, V. Trajković, T.J. Sabo, S. Grgurić-Šipka, I. Marković, Chem. Med. Chem. 6 (2011) 1884–1891.
[36] S. Gómez-Ruiz, D. Maksimović-Ivanić, S. Mijatović, G.N. Kaluđerović, Bioinorg. Chem. Appl. (2012), http://dx.doi.org/10.1155/2012/140284.
[37] M.V. Engeland, L.J. Nieland, F.C. Ramaekers, B. Schutte, C.P. Reutelingsperger, Cytometry 31 (1998) 1–9.
[38] C. Constantinou, K.A. Papas, A.I. Constantinou, Curr. Cancer Drug Targets 9 (2009) 717–728.
[39] Q. Huang, F. Li, X. Liu, W. Li, W. Shi, F. Liu, B. O'Sullivan, Z. He, Y. Peng, A. Tan, L. Zhou, J. Shen, G. Han, X. Wang, J. Thorburn, A. Thorburn, A. Jimeno, D. Raben, J.S. Bedford, C. Li, Nat. Med. 17 (2011) 860–866.