European Journal of Medicinal Chemistry 44 (2009) 3452–3458

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Palladium(II) complexes with R_2edda-derived ligands. Part II. Synthesis, characterization and *in vitro* antitumoral studies of R_2eddip esters and palladium(II) complexes

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ARTICLE INFO

Article history:
Received 20 November 2008
Received in revised form
19 January 2009
Accepted 7 February 2009
Available online 20 February 2009

Keywords:
Palladium(II) complexes
Antitumoral activity
Crystal structure
EDDA-type ligands
DFT calculations

ABSTRACT

New R_2eddip-type esters (R = cyclopentyl, **L3**·2HCl 1.5H_2O; cyclohexyl, **L4**·2HCl·H_2O) and corresponding palladium(II) complexes, [$PdCl_2$**L3**] (**3**) and [$PdCl_2$**L4**]·H_2O (**4**), as well as [$PdCl_2$**L2**] (**2**; **L2** diisobutyl ester of eddip) were synthesized and characterized by IR, ^{1}H and ^{13}C NMR spectroscopies and elemental analysis. The crystal structure of **L3**·2HCl·2$CHCl_3$ was resolved and is given herein. The NMR spectroscopy confirmed the presence of two isomers (from three possible) for each palladium(II) complex. DFT calculations support the formation of two diastereoisomers. In addition, antitumoral investigations were performed and these investigations also included the diisopropyl ester of eddip (**L1**) and corresponding palladium(II) complex, [$PdCl_2$**L1**] (**1**). *In vitro* antiproliferative activity was determined against several tumor cell lines HeLa, Fem-x, K562 and rested and stimulated normal immunocompetent cells (human peripheral blood mononuclear cells -PBMCs) using MTT test.

1. Introduction

The discovery of cisplatin led scientists to synthesize many platinum-based drugs that could potentially be less toxic to healthy tissue [1] and overcome the resistance of some tumors to this drug [2]. Besides cisplatin, carboplatin and oxaplatin are in worldwide clinical use [3].

The success of metallodrugs is closely linked with the proper choice of ligands, as they play a crucial role in modifying reactivity and lipophilicity, in stabilizing specific oxidation states and in imparting substitution inertness [4].

Our investigations focus mainly on the preparation, characterization and antitumor activity of platinum(II) and platinum(IV) compounds with bis(carboxyalkylamino)ethane and propane ligands, and their derivatives [5–7] (Fig. 1A–D). Some of these Pt(IV) complexes with R_2edda-derived ligands showed higher cytotoxicity than cisplatin. In addition a considerably faster kinetic process inducing tumor cell death was observed for these complexes than that observed for cisplatin [8]. As a result of these findings, our studies are currently focused on complexes with $_N\frown_N$ bidentate esters.

Among non-platinum compounds for cancer treatment complexes containing ruthenium, gold and silver [9], even titanium [10] and tin coordination centers have been reported [11]. Dyson and coworkers reported remarkable work on ferrocenoyl pyridine arene ruthenium complexes and about the effect of the cyclopentadienyl ring on the cytotoxicity of ruthenium PTA compounds [12]. Keppler and collaborators besides very valuable work with platinum compounds reported outstanding results working with ruthenium and palladium complexes [9,13]. Gold complexes were also investigated, gold(III) being isoelectronic and isostructural

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doi:10.1016/j.ejmech.2009.02.002

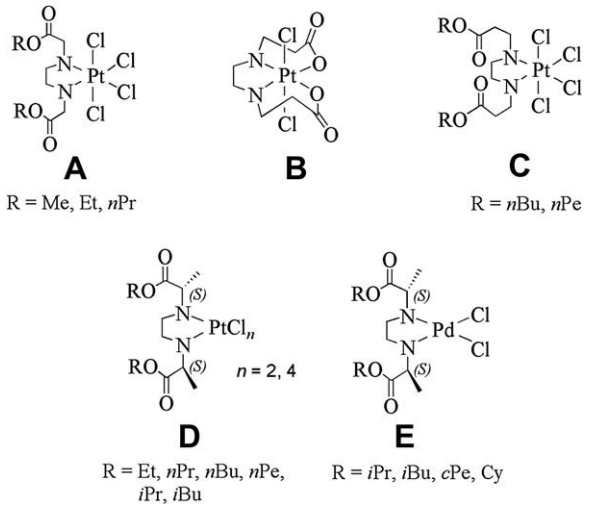


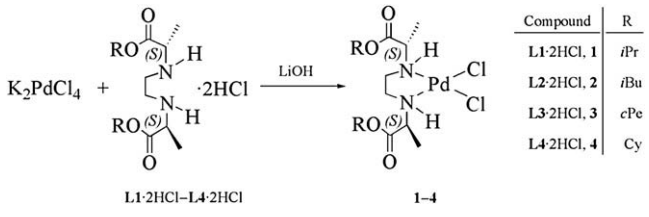
Fig. 1. Platinum and palladium complexes containing R_2edda-derived ligands.

with Pt(II) complexes, and even though most of the compounds show higher lability than platinum(II) compounds, some of the gold(III) compounds have shown great cytotoxicity [14], even gold(I) and silver(I) compounds [15] should be mentioned as possible future anticancer drugs.

Palladium derivatives have also been a subject of wide examination due to their structural analogy with Pt(II) complexes. However, initial results were not very encouraging because the Pd(II) derivatives generally showed lower anticancer activity than cisplatin. This behavior may be related to the more labile nature of palladium(II) relative to platinum(II) complexes [16]. Rapid ligand exchange was thought to diminish the possibility of Pd(II) complexes reaching the biological target unchanged and increase the risk of adverse effects on biochemical processes that occur in normal cells. In order to overcome these problems, several authors [17] have suggested that the use of chelating ligands may reduce the reactivity of the palladium center.

Some Pd(II) complexes were found to be with high cytotoxicity comparable to cisplatin and carboplatin (one of the complexes is found to be 7800 times more active than carboplatin) [18] and it seems that with bulky ligands these complexes exert better antitumoral activity. A number of palladium(II) complexes with neutral ligands such as pyridine derivatives [19], phosphonate derivatives of quinoline [20] or pyrazole derivatives [21] have been investigated and their significant cytotoxic activity has been proved.

Recently, in a reaction of $K_2[PdCl_4]$ with *O*,*O*′-diisopropyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride monohydrate, two products were prepared, one with intact ester groups



Scheme 1. Formation of esters (**L1**·2HCl–**L4**·2HCl) and corresponding Pd(II) complexes (**1**–**4**).

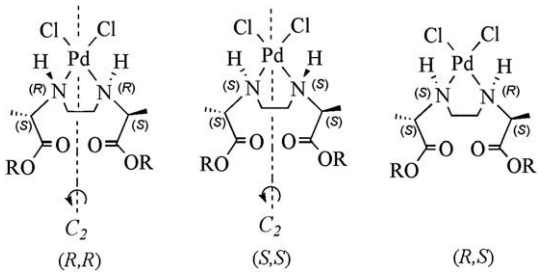


Fig. 2. Diastereoisomers of [$PdCl_2${(*S*,*S*)-R_2eddip}], R = *i*Bu, *c*Pe, Cy.

(Fig. 1E, R = *i*Pr), and a partly hydrolyzed complex [PdCl{(*S*,*S*)-*i*Preddip-$\kappa^2N,N',\kappa O$}] [22]. Herein, we report the synthesis, characterization and antiproliferative activity of two novel R_2eddip-type ligand precursors *O*,*O*′-dicyclopentyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride sesquihydrate, **L3**·2HCl·1.5H_2O, and *O*,*O*′-dicyclohexyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate dihydrochloride monohydrate, **L4**·2HCl·H_2O, their corresponding palladium(II) complexes dichloro(*O*,*O*′-dicyclopentyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate)palladium(II), [$PdCl_2$**L3**], (**3**) and dichloro(*O*,*O*′-dicyclohexyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate)palladium(II) monohydrate, [$PdCl_2$**L4**]·H_2O, (**4**), as well as dichloro(*O*,*O*′-diisobutyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate)palladium(II) [$PdCl_2$**L2**] (**2**), (Fig. 1E). The crystal structure of **L3**·2HCl·2$CHCl_3$ is also reported. DFT calculations were performed on the complexes. In addition, antitumoral investigations were performed including previously obtained complex compound dichloro(*O*,*O*′-diisopropyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoate)palladium(II), [$PdCl_2$**L1**], (**1**).

2. Results and discussion

2.1. Chemistry

Esters **L3**·2HCl and **L4**·2HCl and neutral palladium(II) complexes **2**–**4** were prepared by using appropriate modification of known methods [6–8,23,24]. These esters are not soluble in chloroform, slightly soluble in ethanol and methanol, and fairly soluble in dimethylsulfoxide and in water. Complexes were synthesized by combining aqueous solutions of $K_2[PdCl_4]$ and the corresponding esters (Scheme 1). The resulting complexes are soluble in chloroform and ethanol but not soluble in water, acetone or methanol.

2.2. Spectroscopic measurements

IR spectra of **2**–**4** show specific absorption bands: ν(C=O) at 1740, 1734 and 1734 cm^{-1}, ν(C–O) at 1212, 1223 and 1223 cm^{-1} and $\nu(CH_3)$ at 2960, 2965 and 2940 cm^{-1}; (**L2**·2HCl, **L3**·2HCl, **L4**·2HCl: ν(C=O) at 1743, 1739, 1744 cm^{-1}; ν(C–O) at 1224, 1233, 1224 cm^{-1} and $\nu(CH_3)$ at 2963, 2970, 2940 cm^{-1}). Indication of nitrogen

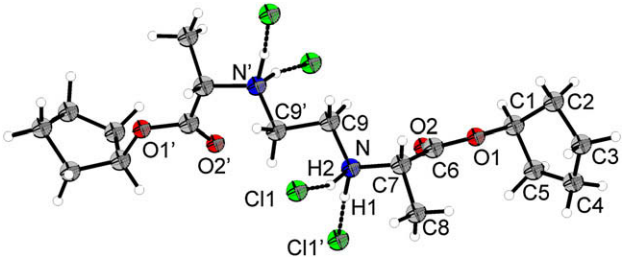


Fig. 3. Molecular structure of **L3**·2HCl in crystals of **L3**·2HCl·2$CHCl_3$.

Table 1
Selected bond lengths (Å) and angles (°) for **L3**·2HCl·2CHCl$_3$.

Bond lengths (Å)		Angles (°)	
O1–C6	1.326(6)	N1–C7–C6	110.1(4)
O1–C1	1.480(7)	C6–O1–C1	116.4(4)
O2–C6	1.196(6)	C5–C1–O1	108.0(6)
N1–C9	1.485(7)	C5–C1–C2	105.8(5)
N1–C7	1.493(6)	C5–C4–C3	109.9(8)
N1–H2N	1.13(5)	H2N–N1–H1N	96(4)
N1–H1N	1.10(2)	C9–N1–H2N	112(3)
C5–C4	1.424(1)	C7–N1–H1N	114(4)

coordination can be proved by the presence of bands for secondary amino groups (3113, 3115 and 3125 cm^{-1}), and none of the complexes has a band for a secondary ammonium group (as found in spectra of ligand precursors **L2**·2HCl, **L3**·2HCl, **L4**·2HCl: $\nu(R_2NH_2^+)$ at 3449, 3415, 3417 cm^{-1}).

In ^{1}H and ^{13}C NMR spectra the expected signals were found. In ^{1}H NMR spectra the broad signals of hydrogen atoms belonging to secondary amino groups appear between 5.8 and 6.6 ppm (comparing with **L2**·2HCl–**L4**·2HCl: 9.5–10.5 ppm). The signals of CH_2 protons of the ethylenediamine bridge show coordination induced shifts (up to 0.9 ppm) giving a clear indication of nitrogen coordination. Signals of cyclopentyl and cyclohexyl protons were found between 1.1 and 1.9 ppm (**L3**·2HCl, **L4**·2HCl, **3** and **4**). Methyl protons from α-alaninato moiety were found at around 1.5 ppm (**L3**·2HCl, **L4**·2HCl, **2**–**4**), and methyl protons from the isobutyl group in the case of **L2**·2HCl and **2** were found at 0.88 ppm. Ester carbon atom resonances were found as expected at around 170 ppm for all compounds verifying that oxygen is not a ligating atom. For the complexes **2**–**4** NMR spectroscopic measurements uphold their constitution. Two sets of signals were found for complexes **2**–**4** indicating the formation of diastereoisomers. Coordination of ligands to the $PdCl_2$ fragment induces formation of two extra chiral centers at ligating N atoms. Thus, three diastereoisomers should be expected for [$PdCl_2${(*S*,*S*)-R_2eddip}] (R = *i*Bu, *c*Pe, Cy; (*R*,*R*), (*R*,*S* ≡ *S*,*R*) and (*S*,*S*), Fig. 2). Two of these three possible diastereomers (*S*,*S*) and (*R*,*R*), will give rise to (due to C_2 symmetry) one set of resonances each for their ester branches. The third diastereoisomer, (*R*,*S*), should give rise to two sets of signals since the ester branches are inequivalent. The stability of all complexes in DMSO solutions was also studied. ^{1}H NMR spectra of all complexes were recorded in deuterated DMSO after 7 days and no evidences of decomposition or evolution to other products were observed suggesting stability of Pd(II) complexes in DMSO.

2.3. X-ray study

The ligand precursor **L3**·2HCl crystallized as **L3**·2HCl·2CHCl$_3$ in the chiral orthorhombic space group $P2_12_12$. Compound **L3**·2HCl·2CHCl$_3$ exhibited crystallographically imposed C_2 symmetry. The molecular structure of **L3**·2HCl·2CHCl$_3$ is shown in Fig. 3. Selected bond lengths and angles are listed in Table 1. All bond lengths are within the expected range and similar to other related compounds [23,25]. The most significant hydrogen bonds in the packing of this molecule are N–H1N···Cl′, 3.120(4) Å, 168(5)° and N–H2N···Cl, 3.095(4) Å, 159(4)°, as shown in Fig. 3.

2.4. Quantum chemical calculations

To determine which two isomers were formed for **2**–**4** quantum chemical calculations were employed. DFT calculations were conducted for the isomers arising from coordination of (*S*,*S*)-R_2eddip, R = *i*Bu, *c*Pe, Cy to $PdCl_2$ fragment. Calculated structures of complexes **2**–**4** (defined as **2c**–**4c**) are shown in Fig. 4. The structures were fully optimized without any symmetry constraints and were found to represent equilibrium structures. The calculated results for all complexes, **2c**, **3c** and **4c** showed that (*R*,*R*) and (*R*,*S*) diastereoisomers appear to be structurally and synthetically feasible. Differences in energy between (*R*,*R*) and (*R*,*S*) isomers are 0.5 (**2c**) and 0.9 kcal/mol (**3c** and **4c**), being within the error of DFT calculations. For all calculated structures the third diastereoisomer (*S*,*S*) is higher in energy than (*R*,*R*), $\Delta E_{(S,S)-(R,R)} = 4.3$–5.4 kcal/mol, and formation of this isomer should not be expected. Results from NMR spectroscopy (within the sensitivity limits of NMR spectroscopy) show the presence of two isomers of **2**–**4** and DFT calculations indicate that they could be assigned as (*R*,*R*) and (*R*,*S*). The introduction of one CH_3 group seems to influence the distribution of isomers compared to related compounds [6,22].

2.5. Cytotoxicity

Esters, **L3**·2HCl and **L4**·2HCl, and palladium(II) complexes **1**–**4** were tested for cytotoxic activity on tumor cell lines human adenocarcinoma HeLa, human myelogenous leukemia K562, human malignant melanoma Fem-x and normal immunocompetent cells, i.e. human peripheral blood mononuclear cells (PBMCs). The analyzed compounds showed a dose-dependent antiproliferative effect towards all cell lines and on human unstimulated and stimulated PBMC. Recently reported cytotoxicities for Pt(II) and Pt(IV) complexes with R_2eddip ligands, as well as cisplatin, are included for comparison (Table 2).

The compound **L3**·2HCl has low to medium activity against the selected cell lines, but in the case of **L4**·2HCl the activity against the cell line K562 is considerably higher ($IC_{50} = 29.8 \pm 3.1$ μM). Compound **L4**·2HCl is quite selective with a low effect on normal and stimulated PBMCs. When the cyclopentyl group is substituted with cyclohexyl, antiproliferative activity on all target cells increases. This is also the case for **3** and **4**. Complex **2** is slightly more active than **1**, so when the isobutyl group is substituted with isopropyl, the cytotoxic action

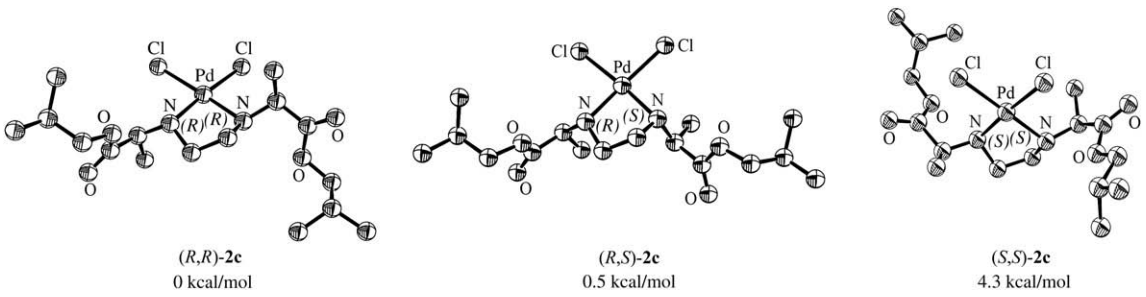


Fig. 4. Calculated structures of [$PdCl_2$**L2**], **2c** (energies are relative to the most stable isomer (*R*,*R*)-**2c**). H atoms omitted for clarity.

Table 2
IC_{50} (μM) for the 72 h of action of investigated compounds on HeLa, K562, Fem-x cells, on PBMC and PBMC stimulated with phytohaemaglutinin (PHA) determined by MTT test.

Compound	HeLa	Fem-x	K562	PBMC	PBMC + PHA
L1·2HCl[a]	>200	>200	>200	>200	>200
L2·2HCl[a]	180.4 ± 7.3	178.0 ± 12.8	180.9 ± 19.1	>200	>200
L3·2HCl	171.5 ± 6.8	89.5 ± 2.9	82.8 ± 1.3	>200	155.0 ± 11.5
L4·2HCl	89.0 ± 3.6	69.9 ± 1.9	29.8 ± 3.1	141.7 ± 1.1	102.7 ± 7.6
1, [$PdCl_2$**L1**]	>200	193.3 ± 1.7	>200	>200	>200
2, [$PdCl_2$**L2**]	>200	139.5 ± 4.6	169.3 ± 3.5	>200	182.9 ± 2.4
3, [$PdCl_2$**L3**]	186.6 ± 1.9	94.7 ± 2.2	92.0 ± 2.8	175.4 ± 2.0	143.9 ± 2.0
4, [$PdCl_2$**L4**]	127.0 ± 7.9	68.0 ± 4.9	61.8 ± 4.4	154.5 ± 2.7	124.9 ± 0.3
[$PtCl_2$**L1**][a]	68.1 ± 4.8	52.5 ± 6.4	29.2 ± 4.7	124.3 ± 15.6	101.2 ± 9.7
[$PtCl_2$**L2**][a]	56.4 ± 8.6	46.0 ± 5.6	23.5 ± 1.4	63.8 ± 2.4	60.6 ± 11.1
[$PtCl_4$**L1**][a]	30.5 ± 2.5	13.7 ± 3.2	12.3 ± 2.6	80.2 ± 24.1	71.3 ± 21.7
[$PtCl_4$**L2**][a]	51.8 ± 9.9	36.0 ± 5.4	26.4 ± 1.0	91.0 ± 6.2	100.6 ± 6.8
Cisplatin	4.5 ± 0.3	4.7 ± 0.3	5.8 ± 0.3	33.6	26.5 ± 5.7

[a] From [23].

decreases. In general, it seems that as the lipophilicity of the ester groups increases (Cy > *c*Pe > *i*Bu > *i*Pr), cytotoxicity becomes higher and this rule may imply for complexes and ligands given in Table 2.

In vitro cytotoxic activities of **L1**·2HCl, **L2**·2HCl, [$PtCl_2$**L1**], [$PtCl_2$**L2**], [$PtCl_4$**L1**], [$PtCl_4$**L2**] and cisplatin are included in Table 2 for comparison [23]. The most active compound reported in this work **L4**·2HCl is twice as active against K562 cells than its complex with palladium(II) (**4**). Comparing with previous work, **L4**·2HCl showed cytotoxicity similar to [$PtCl_2$**L1**] which is about five times less active than cisplatin, yet the more active compound is [$PtCl_4$**L1**] that exerts half of the activity of cisplatin on the K562 cell line. Active platinum compounds such as [$PtCl_4$(*n*Bu_2eddp)] and [$PtCl_4$(*n*Pe_2eddp)] (*n*Bu_2eddp = *O*,*O*′-dibutyl-; *n*Pe_2eddp = *O*,*O*′-dipentyl-ethylenediamine-*N*,*N*′-di-3-propanoate; Fig. 1C; i.e. IC_{50} = 7.1 ± 2.0 and 5.9 ± 2.0 μM against K562, respectively) previously have been shown to have activities comparable with cisplatin, [8]. From the literature it is known that palladium(II) possesses lower cytotoxicity than platinum complexes. When compared with platinum all palladium(II) complexes herein show medium activity (the most active compound **4** is 10 times less active than cisplatin against K562 cells).

The majority of the investigated compounds shows low selectivity between tumor cells and normal blood cells, PBMC. The most selective compound in antitumor action among evaluated palladium(II) complexes is [$PdCl_2$**L4**], against K562 cells (IC_{50}(PBMC)/IC_{50}(K562) = 2.5; IC_{50}(PBMC + PHA)/IC_{50}(K562) = 2.0) but the highest selectivity is observed for its ligand precursor **L4**·2HCl, against the same tumor cell line (IC_{50}(PBMC)/IC_{50}(K562) = 4.7; IC_{50}(PBMC + PHA)/IC_{50}(K562) = 3.4). For comparison, selectivity of cisplatin is IC_{50}(PBMC)/IC_{50}(K562) = 5.8; IC_{50}(PBMC + PHA)/IC_{50}(K562) = 4.6 and IC_{50}(PBMC)/IC_{50}(K562) = 6.5; IC_{50}(PBMC + PHA)/IC_{50}(K562) = 5.8 for [$PtCl_4$**L1**].

3. Conclusions

R_2eddip-type esters and the corresponding palladium(II) complexes were synthesized and characterized. The crystal structure of **L3**·2HCl·2$CHCl_3$ is reported. NMR spectroscopy shows the presence of two diastereoisomeric forms. DFT calculations indicate the most stable diastereoisomers are (*R*,*R*) and (*R*,*S*) for all complexes. The cytotoxic activity of all the synthesized compounds, including the previously synthesized complex [$PdCl_2$**L1**], was established for tumor cell lines human adenocarcinoma HeLa, human myelogenous leukemia K562, human malignant melanoma Fem-x and normal immunocompetent cells, i.e. human PBMC. The esters and their corresponding palladium(II) complexes show moderate cytotoxic activity against selected cell lines, however, this is not comparable with the very high activity of similar platinum complexes. Generally, the palladium complexes have lower cytotoxicity than the corresponding **L1**·2HCl–**L4**·2HCl and similar platinum complexes. The most active ester herein is ligand **L4**·2HCl which gives an IC_{50} value against K562 tumor cell line of 29.8 ± 3.1 μM, while the corresponding palladium(II) complex is **4** with an IC_{50} value of 61.8 ± 4.4 μM on the same cell line. Some structure–activity relationships could be concluded: the order of activity is related to the R substituent (*S*,*S*)-R_2eddip, (R = Cy > *c*Pe > *i*Bu > *i*Pr) for both ligand precursors and palladium(II) complexes. It may be concluded that on raising the lipophilicity of the ester groups (Cy > *c*Pe > *i*Bu > *i*Pr) the cytotoxic activity increases in both, esters and in the palladium complexes. When the central metal ion is changed from Pt(II/IV) to Pd(II) cytotoxic action decreases significantly. In our future work it is planed to increase lipophilicity of the ligands by changing the ester groups as well as the alkyl groups on C7. These manipulations may result in higher cytotoxicity of palladium(II) complexes, thus they might have an application as anticancer drugs.

4. Experimental

4.1. Materials and methods

(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoic acid hydrochloride, [(*S*,*S*)-H_3eddip]Cl, esters **L1**·2HCl·H_2O, **L2**·2HCl·H_2O and the complex [$PdCl_2$**L1**] were prepared by methods described in literature [22,23,26]. $K_2[PdCl_4]$ was purchased from Merck and used without further purification. Infrared spectra were recorded on a Perkin–Elmer FTIR 31725-X spectrophotometer using the KBr pellet technique (4000–400 cm^{-1}). ^1H and ^{13}C NMR spectra were recorded on Bruker Avance 500 (500 MHz) spectrometer in DMSO-d_6 and $CDCl_3$ using tetramethylsilane as internal standard. Elemental analyses for C, H and N were done on a Vario III CHNOS Elemental Analyzer, Elementar Analysensysteme GmbH.

4.2. Chemical synthesis

4.2.1. Synthesis of esters *L3*·2HCl and *L4*·2HCl

These esters were prepared by using the esterification reaction previously described [27]. Thionyl chloride (4.0 ml, 55 mmol) was introduced into a flask containing 50 ml of cyclopentyl or cyclohexyl alcohol (anhydrous conditions) over 1 h. After that 2.0 g (8.31 mmol) of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-propanoic acid hydrochloride, [(*S*,*S*)-H_3eddip]Cl, was added to the flask and the suspension was refluxed for 16 h. The mixture was filtered and the filtrate was left for a few days at 4 °C, yielding the crude product that was filtered off and washed with $CHCl_3$ (3 × 2 ml). A small quantity of crude **L3**·2HCl·1.5H_2O was dissolved in 10 ml of warm cyclopentyl alcohol containing 2 ml of $CHCl_3$ and from this solution after a few days crystals suitable for X-ray analysis were obtained.

L3·2HCl·1.5H_2O, yield: 1.9 g, 52%. Anal. calcd. for $C_{18}H_{34}Cl_2N_2O_4$·1.5H_2O: C, 49.09; H, 8.47; N, 6.36%. Found: C, 48.88; H, 7.87; N, 6.12%. ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 1.35–1.85 (m, 16H, CH_2–*c*Pe), 1.47 (d, 6H, $^3J_{H,H}$ = 7.05, CH_3), 3.42 (m, 4H, C9H_2), 4.16 (m, 2H, C7*H*), 5.17 (m, 2H, C*H*–*c*Pe) 9.80–10.40 (broad s, 4H, NH_2^+). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 14.1 (*C*H_3), 23.2 (*C*H_2–*c*Pe), 32.0 (*C*H_2–*c*Pe), 40.8 (*C*9H_2), 54.3 (*C*7H), 79.0 (*C*H–*c*Pe), 168.7 (*C*OO–*c*Pe). IR (KBr, cm^{-1}): 3408, 2965, 2871, 1741, 1487, 1233, 1130, 960.

L4·2HCl·H_2O, yield: 2.1 g, 55%. Anal. calcd. for $C_{20}H_{38}Cl_2N_2O_4$·H_2O: C, 52.28; H, 8.78; N, 6.10%. Found: C, 51.78; H, 8.74; N, 5.70%. ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 1.18–1.37 and 1.59–1.82 (m, 20H, CH_2–Cy), 1.46 (d, 6H, $^3J_{H,H}$ = 7.06, CH_3), 3.36

(m, 4H, C9H_2), 4.15 (m, 2H, C7*H*), 4.77 (m, 2H, C*H*–Cy), 9.70–10.20 (br s, 4H, NH_2^+). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 14.2 (*C*H_3), 22.8 (*C*H_2–Cy), 24.7 (*C*H_2–Cy), 30.6 (*C*H_2–Cy), 41.0 (*C*9H_2), 54.4 (*C*7H), 74.2 (*C*H–Cy), 168.4 (*C*OO–Cy). IR (KBr, cm^{-1}): 3417, 2937, 2862, 1741, 1459, 1224, 1111, 913.

*4.2.2. Synthesis of complexes **2**–**4***

Complexes were obtained by mixing 10 ml aqueous solution of K_2[$PdCl_4$] (0.158 g, 0.512 mmol) and the respective ligand (**L2**·2HCl·H_2O: 0.194 g, 0.512 mmol; **L3**·2HCl·1.5H_2O: 0.225 g, 0.512 mmol; **L4**·2HCl·H_2O: 0.235 g, 0.512 mmol). During 2 h of stirring 10 cm^3 of 0.1 mol dm^{-3} LiOH (0.040 g, 1.024 mmol) was added in small portions to the reaction solution. Within this period a yellow precipitate was observed, filtered off and air dried.

Compound **2**: Yield 0.233 g, 92%. Anal calcd. for $C_{16}H_{32}Cl_2N_2O_4Pd$: C, 38.92; H, 6.53; N, 5.67%. Found: C, 38.98; H, 6.78; N, 5.74%. Isomer A: ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 0.88 (d, 12H, $^3J_{H,H} = 6.50$, C*H*$_3$–*i*Bu) 1.65 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 1.89 (m, 2H, C*H*–*i*Bu), 2.39 and 2.64 (m, 4H, C9H_2), 3.86 (m, 4H, C*H*$_2$–*i*Bu), 4.26 (m, 2H, C7*H*), 6.45–6.65 (m, 2H, N*H*). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 14.6 (*C*H_3), 18.7 (*C*H$_3$–*i*Bu), 27.0 (*C*H–*i*Bu), 45.7 (*C*9H_2), 55.0 (*C*7H), 70.5 (*C*H$_2$–*i*Bu), 169.7 (*C*OO–*i*Bu). Isomer B: ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 0.89 (d, 12H, $^3J_{H,H} = 6.50$, C*H*$_3$–*i*Bu), 1.43 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 1.89 (m, 2H, C*H*–*i*Bu), 2.44 and 2. 82 (m, 4H, C9H_2), 3.99 (m, 4H, C*H*$_2$–*i*Bu), 4.20 (m, 2H, C7*H*), 5.95–6.15 (m, 2H, N*H*) ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 14.8 (*C*H_3), 18.9 (*C*H$_3$–*i*Bu), 27.1 (*C*H–*i*Bu), 47.2 (*C*9H_2), 56.2 (*C*7H), 70.1 (*C*H$_2$–*i*Bu), 170.7 (*C*OO–*i*Bu). Ratio of isomers A/B = 3/1.

Isomer A: ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 0.91 (d, 12H, $^3J_{H,H} = 7.00$, C*H*$_3$–*i*Bu) 1.62 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 1.97 (m, 2H, C*H*–*i*Bu), 2.52 and 2.87 (m, 4H, C9H_2), 3.90 (m, 4H, C*H*$_2$–*i*Bu), 4.58 (m, 2H, C7*H*), 6.32 (m, 2H, N*H*). Isomer B: ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 0.96 (d, 12H, $^3J_{H,H} = 6.50$, C*H*$_3$–*i*Bu), 1.76 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 1.97 (m, 2H, C*H*–*i*Bu), 2.73 and 3.18 (m, 4H, C9H_2), 3.99 (m, 4H, C*H*$_2$–*i*Bu), 4.19 (m, 2H, C7*H*), 6.20 (m, 2H, N*H*). Ratio of isomers A/B = 2/1.

IR (KBr, cm^{-1}): 3113, 2960, 2880, 1740, 1469, 1212, 1139, 988.

Compound **3**: Yield 0.180 g, 68%. Anal. calcd. for $C_{18}H_{32}Cl_2N_2O_4Pd$: C, 41.75; H, 6.23; N, 5.41%. Found: C, 41.36; H, 6.55; N, 5.21%. Isomer A: ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.50–1.85 (m, 16H, C*H*$_2$–*c*Pe), 1.61 (d, 6H, $^3J_{H,H} = 7.00$, C*H*$_3$), 2.34 and 2.61 (m, 4H, C9H_2), 4.16 (m, 2H, C7*H*), 5.10 (m, 2H, C*H*–*c*Pe) 6.40–6.60 (m, 2H, N*H*). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 14.4 (*C*H_3), 23.2 (*C*H$_2$–*c*Pe), 32.0 (*C*H$_2$–*c*Pe), 45.8 (*C*9H_2), 55.1 (*C*7H), 77.6 (*C*H–*c*Pe), 169.4 (*C*OO–*c*Pe). Isomer B: ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.50–1.85 (m, 16H, C*H*$_2$–*c*Pe), 1.39 (d, 6H, $^3J_{H,H} = 7.00$, C*H*$_3$), 2.43 and 2.80 (m, 4H, C9H_2), 3.90 (m, 2H, C7*H*), 5.17 (m, 2H, C*H*–*c*Pe), 5.90–6.10 (m, 2H, N*H*). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 14.7 (*C*H_3), 23.2 (*C*H$_2$–*c*Pe), 32.0 (*C*H$_2$–*c*Pe), 46.9 (*C*9H_2), 56.4 (*C*7H), 77.2 (*C*H–*c*Pe), 170.4 (*C*OO–*c*Pe). Ratio of isomers A/B = 3/1.

Isomer A: ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.52–1.91 (m, 16H, C*H*$_2$–*c*Pe), 1.59 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 2.40 and 3.19 (m, 4H, C9H_2), 4.49 (m, 2H, C7*H*), 5.20 (m, 2H, C*H*–*c*Pe) 6.33 (m, 2H, N*H*). Isomer B: ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.52–1.91 (m, 16H, C*H*$_2$–*c*Pe), 2.00 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 2.84 and 3.61 (m, 4H, C9H_2), 4.06 (m, 2H, C7*H*), 5.29 (m, 2H, C*H*–*c*Pe), 6.16 (m, 2H, N*H*). Ratio of isomers A/B = 3/1.

IR (KBr, cm^{-1}): 3115, 2965, 2871, 1734, 1459, 1223, 1139, 960.

Compound **4**: Yield 0.121 g, 42%. Anal calcd. for $C_{20}H_{36}Cl_2N_2O_4Pd·H_2O$: C, 42.60; H, 6.79; N, 4.97%. Found: C, 42.42; H, 6.69; N, 4.71%. Isomer A: ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.21–1.52 and 1.66–1.85 (m, 20H, C*H*$_2$–Cy), 1.63 (d, 6H, $^3J_{H,H} = 7.00$, C*H*$_3$), 2.36 and 2.63 (m, 4H, C9H_2), 4.19 (m, 2H, C7*H*), 4.71 (m, 2H, C*H*–Cy), 6.45–6.65 (m, 2H, N*H*). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 15.2 (*C*H_3), 23.5 (*C*H_2–Cy), 25.3 (*C*H_2–Cy), 31.3 (*C*H_2–Cy), 46.4 (C9H_2), 55.8 (*C*7H), 73.6 (*C*H–Cy), 169.8 (*C*OO–Cy). Isomer B: ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.21–1.52 and 1.66–1.85 (m, 20H, C*H*$_2$–Cy), 1.42 (d, 6H, $^3J_{H,H} = 7.00$, C*H*$_3$), 2.44 and 2.80 (m, 4H, C9H_2), 3.93 (m, 2H, C7*H*), 4.80 (m, 2H, C*H*–Cy), 5.90–6.10 (m, 2H, N*H*). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 15.2 (*C*H_3), 23.5 (*C*H_2–Cy), 25.3 (*C*H_2–Cy), 31.2 (*C*H_2–Cy), 46.4 (*C*9H_2), 56.4 (*C*7H), 73.6 (*C*H–Cy), 169.8 (*C*OO–Cy). Ratio of isomers A/B = 2/1.

Isomer A: ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.20–1.56 and 1.68–1.98 (m, 20H, C*H*$_2$–Cy), 1.61 (d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 2.44 and 3.20 (m, 4H, C9H_2), 4.52 (m, 2H, C7*H*), 4.79 (m, 2H, C*H*–Cy), 6.36 (m, 2H, N*H*). Isomer B: ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.20–1.56 and 1.68–1.98 (m, 20H, C*H*$_2$–Cy), 2.01(d, 6H, $^3J_{H,H} = 7.50$, C*H*$_3$), 2.82 and 3.65 (m, 4H, C9H_2), 4.09 (m, 2H, C7*H*), 4.91 (m, 2H, C*H*–Cy), 6.17 (m, 2H, N*H*). Ratio of isomers A/B = 4/3.

IR (KBr, cm^{-1}): 3125, 2940, 2862, 1734, 1459, 1223, 1130, 932.

4.3. X-ray structure determination for **L3**·2HCl·2$CHCl_3$

Data were collected on a CCD Oxford Xcalibur S (λ(Mo-Kα) = 0.71073 Å) using ω and φ scan modes. Semi-empirical absorption correction was performed with SCALE3 ABSPACK [28]. Table 3 lists crystallographic details. The structure was solved by direct methods [29]. Structure refinement was carried out using full-matrix least-squares routines against F^2 with SHELXS-97 [30]. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms at nitrogen were found in the electron density map and all the others were placed in calculated positions with fixed displacement parameters (riding model). All the hydrogens were refined isotropically. The Diamond program has been used for the representation of the structure [31]. Cambridge Crystallographic Data Center, CCDC No. 709833, contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccfdc.cam.ac.uk).

4.4. Computational details

Geometry optimizations were performed with the Gaussian 03 package [32]. All structures were optimized using the MPW1PW91 functional [33]. The SDD basis set for all atoms was employed in the calculations [34]. All systems have been optimized without symmetry restrictions. The resulting geometries were characterized as equilibrium structures by the analysis of the force constants of normal vibrations. Supplementary data associated with quantum chemical calculations can be obtained from the authors upon request.

4.5. Cytotoxicity assay

4.5.1. Preparation of solutions of drugs

Stock solutions of investigated palladium complexes were made in dimethylsulfoxide (DMSO) at a concentration of 20 mM, filtered through Millipore filter, 0.22 μm, before use, and diluted by nutrient medium to various working concentrations. Nutrient medium was RPMI-1640 medium, without phenol red, supplemented with L-glutamine (3 mM), streptomycin (100 mg/ml), and penicillin (100 IU/ml), 10% fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was dissolved (5 mg/ml) in phosphate buffer saline pH 7.2, and filtered through Millipore filter, 0.22 μm, before use. All reagents were purchased from Sigma Chemicals.

Table 3
Crystallographic data for **L3**·2HCl·2CHCl$_3$.

Empirical formula	$C_{20}H_{36}Cl_8N_2O_4$
Mr	652.11
Crystal system	Orthorhombic
Space group	$P2_12_12$
a/Å	14.5059(5)
b/Å	20.0906(1)
c/Å	5.2876(2)
V/Å^3	1540.98(1)
Z	2
D_{calc}/g cm^{-3}	1.405
μ (Mo-Kα)/mm^{-1}	0.759
F(000)	676
θ Range/°	2.7985–32.358
Refln. collected	12,515
Refln. observed [$I > 2\sigma(I)$]	1830
Refln. independent	2929
Data/restraints/parameters	2929/1/174
Goodness-of-fit on F^2	0.907
$R1$, $wR2$ [$I > 2\sigma(I)$]	0.0564, 0.1108
$R1$, $wR2$ (all data)	0.1027, 0.1227
Largest diff. peak and hole/e Å^{-3}	0.529/−0.328

4.5.2. Cell culture

Human cervix adenocarcinoma HeLa and malignant melanoma Fem-x cells were cultured as monolayers in the nutrient medium, while human myelogenous leukemia K562 cells were maintained as suspension culture. The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere. Peripheral blood mononuclear cells (PBMCs) were separated from whole heparinized blood from a healthy volunteer by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g/l gelatine polymers, pH 7.4), were counted and resuspended in nutrient medium.

4.5.3. Cell sensitivity analysis

HeLa and Fem-x cells were seeded (2000 cells per well) into 96-well microtiter plates and 20 h later, after the cell adherence, five different concentrations of investigated compounds were added to the wells. Final concentrations were in the range from 12.5 to 200 μM. Only nutrient medium was added to the cells in the control wells. Investigated compounds were added to a suspension of leukemia K562 cells (3000 cells per well) 2 h after cell seeding, in the same final concentrations applied to HeLa and Fem-x cells. All experiments were done in triplicate. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank. PBMCs were seeded (150,000 cells per well) into nutrient medium or in nutrient medium enriched with (5 μg/ml) phytohaemaglutinin (PHA – Welcome Diagnostics, England) in 96-well microtiter plates and 2 h later, investigated compounds were added to the wells, in triplicates, to five final concentrations, except to the control wells where a nutrient medium only was added to the cells. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank.

4.5.4. Determination of target cell survival

Cell survival was determined by MTT test according to the method of Mosmann [35] and modified by Ohno and Abe [36], 72 h after the drug addition. Briefly, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline) was added to each well. Samples were incubated for further 4 h at 37 °C in humidified atmosphere with 5% CO_2. Then, 100 μl of 10% SDS was added to the wells. Absorbance was measured at 570 nm on the next day. To achieve cell survival (%), absorbance at 570 nm of a sample with cells grown in the presence of various concentrations of agent was divided with absorbance of control sample (the absorbance of cells grown only in nutrient medium), having subtracted from absorbance of a corresponding sample with target cells the absorbance of the blank.

Acknowledgements

The author G.N. Kaluđerović gratefully acknowledges financial support from the Alexander von Humboldt Foundation. The authors are grateful to the Ministry of Science and Technological Development of the Republic of Serbia for financial support (Grant Nos. 142010 and 145006). We thank Mairéad E. Kelly (Universität Halle – Wittenberg) for helpful discussions.

Appendix A. Supplementary information

Supplementary information for this manuscript can be downloaded at doi:10.1016/j.ejmech.2009.02.002.

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