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ORIGINAL PAPER

Platinum(II)-oxalato complexes of seliciclib (CYC202) derivatives show different cellular effects and lesser adverse effects in mouse lymphoma model than cisplatin

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Abstract

This work presents a deeper pharmacological evaluation of two formerly prepared and characterized, and highly in vitro cytotoxic platinum(II) oxalato complexes [Pt(ox)(L_1)$_2$] (**1**) and [Pt(ox)(L_2)$_2$] (**2**), containing the derivatives of cyclin-dependent kinase inhibitor (CDKi) seliciclib ((*R*)-roscovitine, CYC202) coordinating as *N*-donor carrier ligands, i.e., 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(4-methoxybenzyl)-9-isopropyladenine (L_1) and 2-chloro-*N*6-(2,4-dimethoxybenzyl)-9-isopropyladenine (L_2). The positive results of in vitro cytotoxicity screening on human cancer cell lines (HeLa, HOS, A2780, A2780R, G361 and MCF7 with IC_{50} at low micromolar levels) published previously, motivated us to perform extended preclinical in vitro experiments to reveal the mechanisms associated with the induction of cancer cell death. In addition, the in vivo antitumor activity was evaluated using the mouse lymphocytic leukaemia L1210 model. The obtained results revealed that complex **1** exceeds the antitumor effect of cisplatin (as for the extension of life-span of mice) and shows far less adverse effects as compared to reference drug cisplatin. The in vitro and ex vivo studies of cellular effects and molecular mechanisms of cell death induction showed that the mechanism of action of complex **1** is essentially different from that of cisplatin. The obtained results showed a possible way how to obtain antitumor active platinum(II) oxalato complexes with better therapeutic profile than contemporary used platinum-based therapeutics.

Keywords Platinum(II) complexes · Seliciclib derivatives · Antitumor activity · In vivo · Ex vivo

Abbreviations

Casp	Caspase
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
PBS	Phosphate-buffered saline
PI	Propidium iodide
Ctrl	Control

Introduction

The platinum(II) complexes are the most successful group of the transition metal-based chemotherapeutics, thanks to the cisplatin, carboplatin, oxaliplatin, nedaplatin, lobaplatin and heptaplatin, used for the treatment of various cancer types [1]. There is, however, an everlasting problem associated with the application of platinum-based pharmaceuticals, which is based on toxic effects of these compounds in various organ systems, such as severe loss of appetite or alopecia up to severe neurotoxicity, renal toxicity and hepatotoxicity. Although many novel platinum and non-platinum complexes have been reported in the literature since the discovery of cisplatin, just a few of them were able to show significant improvement of the antitumor activity based on in vivo studies. The studied platinum(II) oxalato complexes [Pt(ox)(L_1)$_2$] (**1**) and [Pt(ox)

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$(L_2)_2$] (**2**), where L_1 = 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(4-methoxybenzyl)-9-isopropyladenine and L_2 = 2-chloro-*N*6-(2,4-dimethoxybenzyl)-9-isopropyladenine, were previously reported by our research group as members of new group of platinum(II) complexes involving the derivatives of well-known cyclin-dependent kinase 2 (CDK2) inhibitor (*R*)-roscovitine (seliciclib, CYC202, see Fig. 1) as *N*-donor ligands [2–4].

These complexes showed moderately high in vitro cytotoxicity against several types of human cancer cell lines than the reference drug cisplatin. Complex **1** showed the promising cytotoxicity towards cervix carcinoma HeLa (IC_{50} = 23.4 µM), osteosarcoma HOS (IC_{50} = 20.4 µM), ovarian carcinoma A2780 (IC_{50} = 14.4 µM), cisplatin-resistant ovarian carcinoma A2780R (IC_{50} = 18.7 µM), malignant melanoma G361 (IC_{50} = 15.8 µM), and breast adenocarcinoma MCF7 (IC_{50} = 26.9 µM) cell lines, exceeding in most of the cases that of cisplatin [3, 4]. Complex **2** was even more in vitro cytotoxic on some of the cell lines as compared with complex **1**, with the IC_{50} values of 27.5 µM (HeLa), 4.0 µM (A2780), 4.1 µM (A2780R), 5.5 µM (G361), 5.4 µM (HOS), and 3.6 µM (MCF7), proving it to be up to seven times more active as compared with cisplatin [2–4].

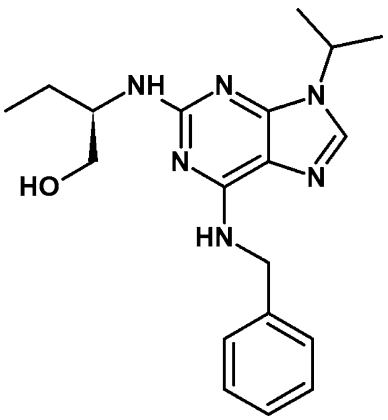

Fig. 1 Structural formula of (*R*)-roscovitine (seliciclib, CYC202)

The development of complexes **1** and **2** was motivated by the clinical success of the platinum(II) complexes (such as oxaliplatin, nedaplatin, lobaplatin or heptaplatin) containing various types of *N*-donor carrier ligands and allowing them to be active against wider spectrum of tumor types (e.g. colorectal carcinoma in case of oxaliplatin). Their application is also connected with less adverse effects than those known for cisplatin (in the case of oxaliplatin, the only dose-limiting factor is neurotoxicity). Furthermore, oxaliplatin is usually not cross-resistant with cisplatin and its diammineplatinum(II) analogues (carboplatin, nedaplatin), and it is effective against the tumors with acquired resistance to cisplatin [5].

With respect to the given facts and based on the positive findings regarding two previously reported platinum(II) oxalato complexes in connection with their in vitro cytotoxicity [2–4], we decided to study more deeply the complexes of the composition [Pt(ox)$(L_1)_2$] (**1**) and [Pt(ox)$(L_2)_2$] (**2**) (Fig. 2) by more advanced in vitro studies, evaluating their cellular and molecular effects against the selected cancer cells, and by the in vivo and ex vivo studies revealing their antitumor activity on the mice bearing the L1210 lymphocytic leukaemia.

Materials and methods

Chemicals

The used chemicals and solvents were supplied by VWR International (Stříbrná Skalice, Czech Republic),

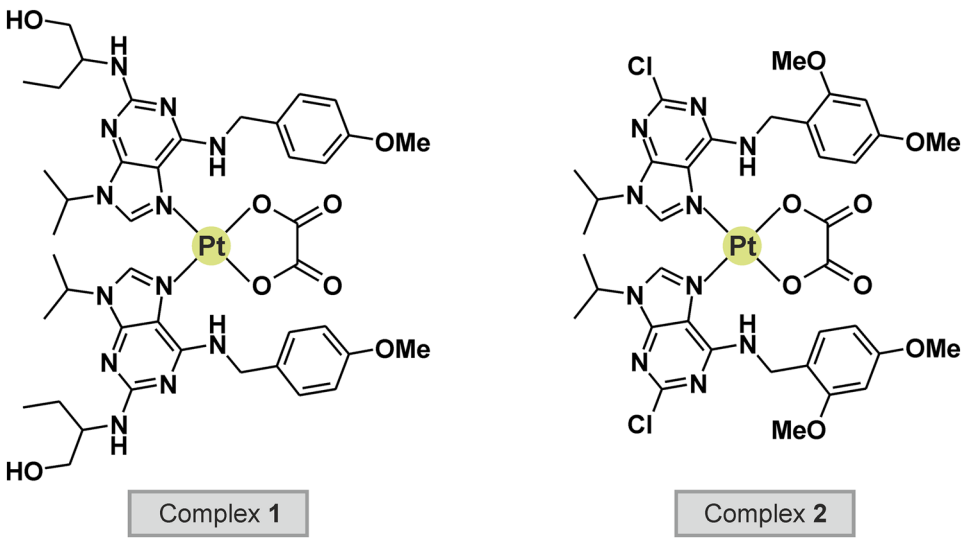

Fig. 2 Structural formulas of the platinum(II) oxalato complexes [Pt(ox)$(L_1)_2$] (**1**) and [Pt(ox)$(L_2)_2$] (**2**); L_1 = 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(4-methoxybenzyl)-9-isopropyladenine and L_2 = 2-chloro-*N*6-(2,4-dimethoxybenzyl)-9-isopropyladenine

Sigma-Aldrich (Prague, Czech Republic), Fisher Scientific (Pardubice, Czech Republic) and Litolab (Chudobín, Czech Republic). The synthetic intermediates $K_2[Pt(ox)_2]\cdot 2H_2O$, 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(4-methoxybenzyl)-9-isopropyladenine (L_1) and 2-chloro-*N*6-(2,4-dimethoxybenzyl)-9-isopropyladenine (L_2), as well as the studied complexes $[Pt(ox)(L_1)_2]$ (**1**) and $[Pt(ox)(L_2)_2]$ (**2**) were prepared as reported previously [2, 3].

Cell cultures

The A2780 human ovarian carcinoma and MCF7 human breast adenocarcinoma cell lines were supplied by the European Collection of Cell Cultures (ECACC). The cells were cultured as adherent monolayers according to the supplier instructions, in particular in RPMI-1640 medium supplemented with 10% of foetal calf serum, 1% of 2 mM glutamine, and 1% penicillin/streptomycin (37 °C and 5% CO_2, humidified incubator).

Cell cycle analysis

The cultured A2780 and MCF7 cells were seeded to the 6-well plates (1×10^6 cells per well) and pre-incubated for 24 h at 37 °C and 5% CO_2 atmosphere. Complexes **1** and **2** (and cisplatin for comparative purposes) were added at the equipotent concentrations (equal to their IC_{50} values at the respective cell type) to the cells. After 24 h exposure time, the cells were harvested using trypsin, washed and fixed with PBS. The cell pellets were resuspended in aqueous solution of RNase A (100 µg/mL) and a mixture of propidium iodide (500 µg/mL) containing surfactant Triton X-100 (0.1% v/v) after PBS removing. The samples were incubated for 30 min at 25 °C in the dark and DNA content was assessed using flow cytometry (CytoFlex, Beckman Coulter, Prague, Czech Republic) detecting emission of DNA-bound PI (maximum at 617 nm, 610/20 BP) after excitation by the blue laser (488 nm). The data were analysed using marker set within the analysis CytExpert™ software (Beckman Coulter, Prague, Czech Republic). Untreated A2780 and MCF7 cells were used as a negative control. The experiments were conducted in triplicate.

Induction of apoptosis

The samples were prepared from ca 1×10^6 cultured A2780 and MCF7 cells, treated by complexes **1**, **2** and cisplatin (24 h exposure time, IC_{50} concentrations), following the instructions supplied in the used Annexin V-FITC Apoptosis Detection Kit (Enzo Life Sciences, Inc., Farmingdale, New York, USA). For more detailed information, see Experimental details in Supplementary material.

Caspase 3/7 activation

The induction of specific cell death type in A2780 and MCF7 cells was analysed by means of the CellEvent™Caspase-3/7 Green Flow Cytometry Assay Kit (Invitrogen, Waltham, Massachusetts, USA). The cells were treated in a similar manner as described above and further processed and results analysed according to the manufacturer's instructions. For more detailed information, see Experimental details in Supplementary material.

In vivo antitumor activity

The in vivo study of anticancer activity was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and the national Act on the Protection of Animals Against Cruelty No. 246/1992 Coll. The Expert Committee on the Protection of Animals Against Cruelty at the University of Veterinary and Pharmaceutical Sciences Brno approved the experimental plan of this study under the permit number 52/2012. The number of pharmacological interventions and manipulations were limited to the necessary minimum to minimize the suffering of laboratory animals. The animals were observed regularly (in ca 2 h intervals) for any signs of unnecessary suffering from the symptoms of the tumor progression. The humane endpoint protocol was based on the evaluation of the following symptoms: (a) symptoms of acute toxicity of tested compounds (observed within 2 h after the injection of the tested compound), (b) weight loss higher than 30% of the initial weight, (c) excessive volume of the tumors hindering the mobility or social communication of the animals. The animals reaching the humane endpoint parameters in at least one of the symptoms were sacrificed immediately by cervical dislocation.

The ability of tested Pt(II) complexes to inhibit the tumor growth (induced by the i.p. injection of L1210 cells) in vivo was evaluated on the male DBA/2 SPF mice as was described previously [6, 7]. The tested mice were housed in the Sealsafe NEXT—IVC Blue Line Housing System (Tecniplast, Italy) to ensure the optimal experimental conditions and eliminate the risk of possible inter-group cross-contamination. Due to the lack of toxicological data and to eliminate the excessive use of laboratory animals, the experimental setup, involving the use of the same dose for all the platinum(II) complexes (3 mg/kg) was administered daily for 5 days. Obtained data, describing the survival of the experimental animals, were expressed as the percentage of mean survival time, %T/C defined as the ratio of the mean survival time of the treated animals (T) divided by the mean survival of the untreated control group (C). There were no sudden deaths attributable to acute toxicity of the tested compounds.

Histological and histochemical evaluations

Biological material obtained by the dissection of the sacrificed animals was divided into two parts, the first one was kept below −80 °C for further evaluation by the methods of molecular biology, and the second one was processed by the histological procedures, as was described previously [6, 7]. The paraffin embedded preparations were stained by the standard haematoxylin and eosin staining. The immunohistochemical detection of the caspases 3 and 8 was performed using the appropriate antibodies obtained from AbCam (Cambridge, UK). The quantification of the tissue expression of caspases in the samples was semi-quantitative. The scale from 0 to 4 was used, where 0 = protein was not detected, 1 = up to 25%, 2 = up to 50%, 3 = up to 75% and 4 = up to 100% of the area contains the detected protein within the view field.

Protein expression analysis

Frozen tumor samples were prepared for immunodetection and analysed as was described previously [6]. For more detailed information, see Experimental details in Supplementary material.

Statistical analysis

The significance of the differences between the results was assessed by the ANOVA analysis, followed by Tukey's post hoc test for multiple comparisons (QC Expert 3.2, Statistical software, TriloByte Ltd., Pardubice, Czech Republic). The significant statistical difference was considered when $p < 0.05$.

Results and discussion

Cell cycle analysis

Flow cytometric studies of cell cycle with propidium iodide (PI) staining (Fig. 3, Table S1 in Supplementary material) demonstrated that exposure of A2780 and MCF7 cells to complex **1** (IC_{50} concentration of 24.0 μM for A2780 cells, and 30.7 μM for MCF7 cells) causes a significant increase of the G2/M cell cycle phase population as compared to cisplatin. In particular, ca 30 and 43% of the detected A2780 and MCF7 cells, respectively, showed at the G2/M region, which is higher than observed for the untreated cells (i.e., negative control) with ca 21% (for A2780) and 23% (for MCF7) cells. This behavior mimics the cell cycle modification pattern of (*R*)-roscovitine (seliciclib, CYC202) as reported in MCF7 cells, which relates to its significant inhibitory effect of this compound towards CDK2 [8]. Unlike for cisplatin, the G2/M cell cycle arrest is also a known mechanism of action for oxaliplatin in MCF7 cells [9]. In comparison with cisplatin (applied at the IC_{50} concentrations of 24.7 μM for A2780 cells and 21.4 μM for MCF7 cells), complex **2** (applied at the IC_{50} concentrations of 3.1 μM for A2780 cells and 5.5 μM for MCF7 cells) did not cause significant cell cycle modifications in A2780 cells, while the modification in MCF7 cells can be considered as significant (Fig. 3). The small increase in the sub-G1 cell population induced by complex **2** might indicate the induction of necrosis in A2780 cells.

Induction of apoptosis

Further flow cytometric studies were performed on A2780 cells by use of Annexin V-FITC Apoptosis detection kit. Complex **1** induced the early stages of apoptosis in A2780 cells (8.1% ± 4.1%) in the similar manner as it was induced by cisplatin (9.2% ± 1.7%). Complex **2** revealed the induction toward the late stages of apoptosis (11.3% ± 5.2%) with less effectivity as compared to cisplatin (26.4% ± 3.0%).

Apoptosis and necrosis caused by complexes **1** and **2** on A2780 cells were studied using Caspase-3/7 kit (see Fig. S1). Complex **2** revealed similar behaviour as cisplatin in induction of apoptosis and necrosis (52.9% ± 0.8% and 30.4% ± 2.2% for complex **2**, respectively, and 63.6% ± 5.1% and 27.5% ± 8.5% for cisplatin, respectively). The highest apoptotic population was observed in case of complex **1** (73.3% ± 0.2%) in comparison to necrotic one (9.5% ± 1.2%). These highly positive results prompted us to test both complexes further in an animal model.

In vivo anticancer activity: survival extension method

The in vivo testing was performed in mice bearing the L1210 mouse lymphocytic leukaemia by a standard survival extension method. The platinum(II) complexes (**1** and **2**), and cisplatin (used as a reference drug), were applied in the same dosing regimen based on 3 mg/kg i.p. application for 5 consecutive days, 10 days after the bolus dose of 2×10^6 L1210 cells was injected i.p. to induce the tumor growth in mice. The survival extension was evaluated as %T/C, where T represents the average survival (in days) for the respective treated group and C represents the average survival (in days) for the control group.

The effectivity of complex **2** to extend the survival of the L1210-bearing mice was at the same level as for cisplatin (%T/C = 93.3%), while complex **1** excelled over these two compounds significantly with the %T/C = 108.7% (Table 1). According to the previously published data, oxaliplatin revealed the %T/C = 211% applied in the dose of 3.12 mg/kg on the days 1, 5, and 9 after the induction of tumorigenesis induced by the i.p. injection of 1×10^5 L1210 cells in

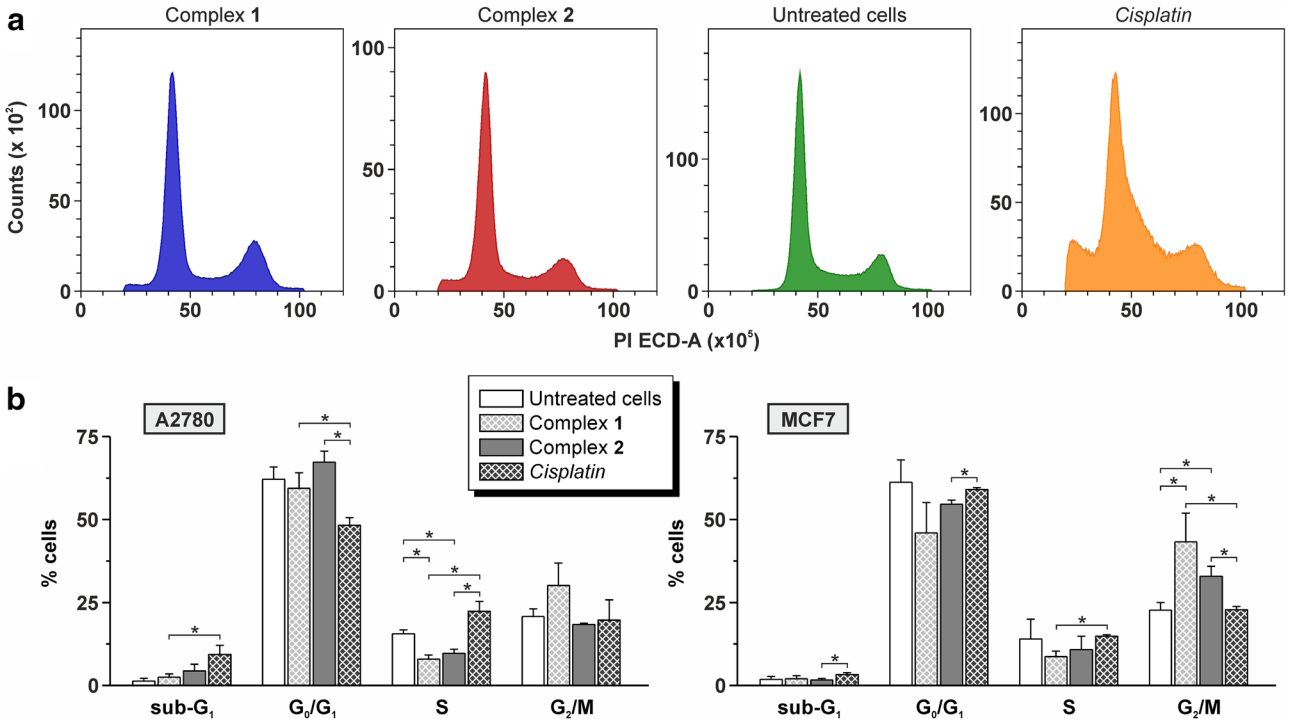

Fig. 3 The selected flow cytometry histograms showing the distribution of A2780 cells stained by propidium iodide (PI) (**a**); the cycle phase populations detected by flow cytometry in A2780 and MCF7 cell populations (the data are given as arithmetic mean from three independent experiments) (**b**). For **a** and **b**-cells were treated for 24 h by the IC_{50} concentrations of complexes **1** and **2** (and cisplatin for comparative purposes). The statistically significant differences ($p < 0.05$) calculated for complexes **1** and **2** against both controls (i.e., untreated cells and cisplatin) are labelled with asterisks (*)

Table 1 Selected parameters characterizing the survival of the mice in L1210 antitumor activity model

Compound	Untreated control	**1**	**2**	Cisplatin
Average body weight ± SE (g)	24.7 ± 1.3	21.8 ± 1.2	22.4 ± 0.7	14.8 ± 0.4
Average tumor weight ± SE (g)	5.1 ± 0.6	5.0 ± 0.3	5.0 ± 0.46	1.5 ± 0.1
Mean survival time ± SE (days)	18.2 ± 0.8	19.8 ± 1.9	17.0 ± 1.2	17.0 ± 1.4
% T/C (%)	100.0	108.7	93.3	93.3

CDF1 mice on day 0 (i.e., a regimen without a 10-day tumor induction phase) [10].

The overall progress of the tumor treatment differed quite significantly between complexes **1** and **2**, and cisplatin-treated group. Cisplatin caused a dramatic decrease of the body weight (in average up to 30% of the initial body weight) and also significant decrease of the tumor tissue weight. However, as a consequence of the cisplatin treatment, the severe adverse effects were observed, such as signs of total systemic failure, apathy, loss of appetite, tachypnoea, and generalized tremor. On the other hand, the mice treated with complex **2** and mainly with complex **1** were much more vivid with no eating disorder, or symptoms of neurological disorder.

The results of the histological evaluations of the tissue samples obtained from the mice sacrificed due to ethical reasons, confirmed the observed relatively low toxicity of complexes **1** and **2** on the healthy cells, because they induced lower extent of various negative organ effects (e.g. cirrhosis, colon endothelial damage, nephrotoxicity). Moreover, these effects can be, from the histological point of view, considered as reversible, while in the case of more toxic cisplatin, the histological damage was deemed as irreversible. From the point of view of welfare, the lower toxicity of complexes **1** and **2** and less aggressive side-effects against cells might be considered as more beneficial for the treated animals than far more deleterious effects of cisplatin. This can also mean that complexes **1** and **2** differ quite significantly from cisplatin in the pharmacodynamic and pharmacokinetic profiles, and therefore, the applied dosing regimen might not be ideal for these complexes to obtain the best curative performance.

To reveal the cellular effects of complexes **1** and **2** in the tumor tissues, the immunohistochemical detection of the selected proteins (see Table S2 and Fig. S2) associated with cell death and evaluation of signs of tissue damage were performed semi-quantitatively. The scale from 0 to 4 was used, where 0 = without detection, 1 = up to 25%, 2 = up to 50%, 3 = up to 75% and 4 = up to 100% of positive cells (or areas showing the certain feature) in the view field. Sections for this evaluation were stained by standard haematoxylin and eosin staining and observed at 200× magnification. In addition, the mitotic index (number of mitoses in the view field at 200× magnification) and reactivity index (the ratio between the number of mitoses and cells in apoptosis in the view field at 200× magnification) were enumerated as useful indicators of the antimitotic and pro-apoptotic action of the studied compounds.

The immunohistochemical evaluation of caspase expression in the tumor tissues showed an interesting feature of complexes **1** and **2** which, unlike cisplatin and control, decreased the expression of caspase 8, while the expression of effector caspase 3 increased significantly. The overall effect of the tested compounds on the tumor tissues, as demonstrated by the value of the reactivity index, which correlates well with the average weight of the tumor tissues, indicates that alongside cisplatin, complex **1** might be considered an effective therapeutic agent, moreover, showing less severe side effects as compared with cisplatin.

Ex vivo evaluation of the expression of selected proteins

A level of the effector caspase 3 (Casp-3), and regulatory protein p53 was measured in tumor tissues obtained from the pharmacological experiment (Fig. 4). In comparison to cisplatin, tested complexes were able to slightly increase the amount of full-length (32 kDa) pro-Casp-3 by 6% (**1**) and 28% (**2**) as compared with the control, while cisplatin decreased its level by 34%. On the other hand, all tested complexes and cisplatin elevated the levels of its cleaved, active form (17 kDa) by 11–34%. Similar results were observed in our previous study, where the platinum(II) complexes involving 7-azaindole derivatives were also able to

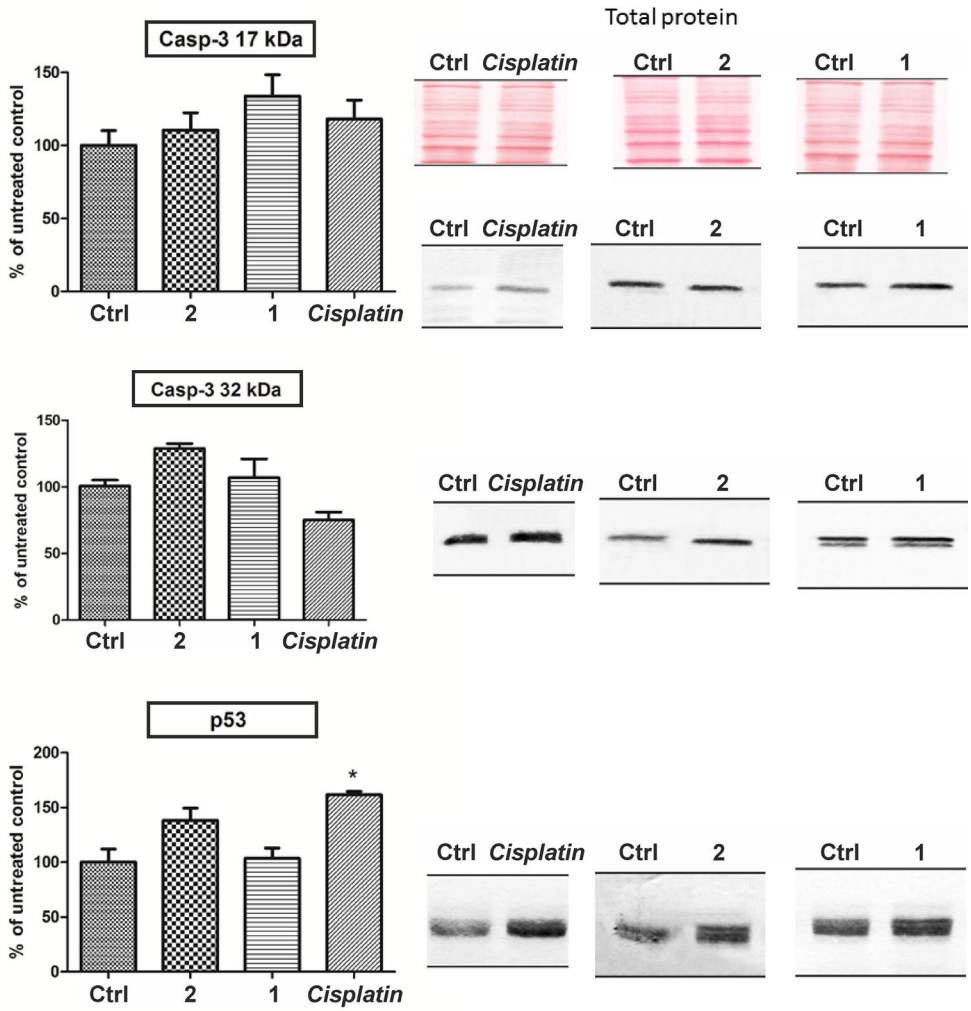

Fig. 4 The amount of the active (17 kDa) and full-length (32 kDa) form of caspase 3 (Casp-3) and amount of p53 protein were detected by Western blot analysis and immunodetection. The results are presented as mean ± SE *Significant difference in comparison to untreated cells (L1210) ($p < 0.05$)

augment the activation of Casp-3 [6]. The activation of the effector Casp-3 is connected with the late stages of apoptosis [11] and thus its higher level leads to tumor growth inhibition.

It is a well-accepted fact that the main mechanism of action of most platinum(II) complexes is connected with the damaging of genomic DNA. This damage triggers the activation of transcription factor p53 and, in case the DNA is damaged beyond repair, it leads to cells death via induction of apoptosis [12]. Complex **2** and cisplatin were able to increase the level of p53 by 38%, and 61%, respectively (Fig. 4). Complex **1** did not affect the p53 level; however, it was able to activate Casp-3. The similar behaviour was observed previously for platinum(II) complexes containing the 7-azaindole derivatives [6]. This might indicate that this complex is able to bypass the main signaling net involved in the cisplatin action and to activate alternative pathways, e.g. induction of mitochondrial dysfunction or autophagy [13].

Conclusions

Following the previously reported positive results of in vitro cytotoxicity screening of the two platinum(II) complexes [Pt(ox)(L_1)$_2$] (**1**) and [Pt(ox)(L_2)$_2$] (**2**) containing either 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(4-methoxybenzyl)-9-isopropyladenine (L_1) or 2-chloro-*N*6-(2,4-dimethoxybenzyl)-9-isopropyladenine (L_2) [2–4], which showed significant in vitro cytotoxicity against a panel of human cancer cell lines (A2780, A2780R, HOS, MCF7, HeLa, G361) and exceeded the cytotoxic potency of cisplatin against these cell lines, we decided to perform a series of advanced in vitro experiments (cell cycle modification, apoptosis induction, caspase 3/7 activation) and evaluate their in vivo antitumor activity using the mouse lymphocytic leukaemia L1210 model. The in vivo action of complexes **1** and **2** was evaluated on whole organism level (survival extension), the overall health condition upon the therapy with the specific compound (systemic and organ toxicity), and on cellular level by immunohistochemical (induction of caspase 3/8 and reactivity index) and Western blot analyses of tumor tissues (post mortem). The overall in vivo antitumor effect of the tested compounds, as demonstrated by the value of the reactivity index, which correlates well with the average weight of the tumor tissues, indicates that alongside cisplatin, complex **1** might be considered an effective tumor suppressing agent, moreover, showing less severe side effects as compared with cisplatin. In addition, the mechanism of its action differs from that of reference drug cisplatin. Taken together, the obtained results pointed out the potential of platinum(II) oxalato complexes involving the seliciclib derivatives as prospective therapeutics with better therapeutic profile as conventional platinum-based therapeutics.

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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

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