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Novel octahedral Pt(IV) complex with di-*n*-propyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoato ligand exerts potent immunomodulatory effects

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ABSTRACT

We have recently reported that a novel octahedral Pt(IV) complex with di-*n*-propyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoato ligand has a potent cytotoxic effect on glioma, melanoma and fibrosarcoma cell lines. In this work, we investigated the influence of the Pt(IV) compound on immune cells. We determined its effect on the viability of spleen cells and lymph node cells and on their capability to produce interferon (IFN)-γ and interleukin (IL)-17. Also, we researched the compound's impact on peritoneal macrophages and generation of NO in these cells. Our results show that the complex has limited influence on cell viability of immune cells, but profound inhibitory effect on the production of examined immune mediators. These results are valuable as they show that the novel Pt(IV) complex applied in concentrations which are effective against tumor cells do not affect immune cell viability. Moreover, they also imply that the complex has immunomodulatory properties.

1. Introduction

Cisplatin (CP) is a platinum(II)-based anticancer drug that has been considered as one of the most effective chemotherapeutic agents, displaying clinical activity against a wide variety of solid tumors [1]. Still, major limitations of CP-based chemotherapy include acquired resistance of some tumors, and the induction of cumulative and irreversible toxicities against non-transformed tissues, including nephrotoxicity, ototoxicity and peripheral neuropathy [1–3]. In order to generate more potent and more selective antitumor drugs, numerous Pt(IV) complexes were synthesized in recent years. Structure-activity relationship investigations of such complexes implied that high lipophilicity increases anticancer activity of these complexes [4], as well as the ease of reduction of Pt(IV) to Pt(II) in the presence of a biological substrate. Importantly, the reduction occurs more readily in the complexes with chlorido than carboxylato axial ligands [5]. Furthermore, in the case of axial chlorido ligands lipophilicity of complexes is preserved by substitution of diamine ligands with large and voluminous aliphatic groups [6,7]. Also, Pt(IV) complexes with esterified ethylenediamine-*N*,*N*′-dipropanoate ligands are potent against tumors, and it has been shown that their anticancer activity depends on the number of carbon atoms on the ester chain, e.g. *n*-propyl esterified ethylenediamine-*N*,*N*′-diacetate ligand exhibiting the most potent cytotoxic efficiency [8,9]. Furthermore, the presence of cyclic amine ligand was shown to increase lipophilicity of platinum complexes, and to facilitate transport of these complexes across the cellular membrane [10]. Thus, accordingly, we have recently synthesized a Pt(IV) complex with di-*n*-propyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoato ligand (L) [11]. This complex - $[Pt(L)Cl_4]$ has two axial chlorido ligands capable for potential reduction in cells. It also contains a cyclic substituent (cyclohexyl group) and *n*-propyl chain in esterifed part of di-*n*-propyl-(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoato ligand that are responsible for enhancing lipophilicity. L coordinates as a bidentate ligand. We have previously reported that $[Pt(L)Cl_4]$ exerts potent *in vitro* activity against several tumor cell lines, i.e. that the IC_{50} value of this complex was lower than IC_{50} value of CP in each of the cell lines tested [11]. In this work, we investigated the effect of the compound on immune cells. Results indicated that the complex applied in concentrations which were effective against tumor cells did not affect immune cell viability. Moreover, higher concentrations of $[Pt(L)Cl_4]$ exerted an immunomodulatory effect.

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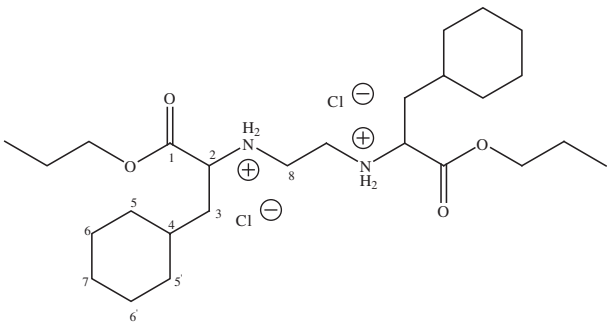
2. Chemistry

2.1. Chemicals and elemental analyses

The ligand dihydrochloride, (*S,S*)-ethylenediamine-*N,N′*-di-2-(3-cyclohexyl) propanoate dipropyl ester dihydrochloride, and the corresponding platinum(IV) complex were prepared as earlier described [11]. The ester crystallizes as a white powder. This compound is well soluble in dimethylsulfoxide (DMSO) but not in water, ethanol, acetonitrile or chloroform. (0.76 g, 53.85%) mp = 216 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.93 (m, *J* 7.2 and 7.4 C***H***$_3$CH$_2$–, 6H; C7, 4H), 1.16 (m, C5, C5′, 8H), 1.43 (m, *J* 6.8 and 7.2, C6, C6′, 8H), 1.63 (m, C***H$_2$***–Cy; C4, 6H; CH$_3$C***H$_2$***–, 4H), 3.58 (m, –C***H$_2$***–OOC–, 4H), 4.16 (m, *J* 6.8, 4.0 and 3.2, –OOC–C***H***–NH$_2$–, 2H, –NH$_2$–C***H$_2$CH$_2$***–NH$_2$–, 4H), 9.78 ppm (m, N**H**$_2^+$, 4H). ^{13}C NMR (50 MHz, [D6]DMSO): 10.5 (**C**H$_3$CH$_2$CH$_2$OOC–), 21.6 (CH$_3$**C**H$_2$CH$_2$OOC–), 26.0 (**C6**), 26.0 (**C4**), 32.0 (**C7**), 33.5 (**C5**), 37.0 (**C3**), 57.5 (**C8**), 67.7 (**C2**, CH$_3$CH$_2$**C**H$_2$OOC–), 169.9 ppm (**C1**); IR (ATR): $\bar{\nu}$ = 2900–2500, 1735, 1451, 1215, 829. ESI/MS/TOF: *m/z* = 453.37 [M–2HCl + H]$^+$. Anal. calcd for $C_{26}H_{50}O_4N_2Cl_2 \cdot 0.5H_2O$: C, 58.91; H, 9.62; N, 5.24, found: C, 58.91; H, 9.89; N, 5.19. The [Pt(L)Cl$_4$] complex crystallizes as yellow powder. The synthesized complex is well soluble only in DMSO. (0.14 g, 53.58%) mp = 276 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.92 (m, *J* 1.8, 1.6, and 7.3, C***H***$_3$CH$_2$–, 6H, C7, 4H), 1.16 (m, C5, C5′, 8H), 1.40 (m, C6, C6′, 8H), 1.64 (m, *J* 5.6 and 6.8, C3; C4, 6H; CH$_3$C***H$_2$***–, 4H), 3.50 (m, –C***H$_2$***–OOC–, 4H), 4.16 (m, *J* 3.4, 6.8 and 7.8, –OOC–C***H***–NH–, 2H, –NH–C***H$_2$CH$_2$***–NH–, 4H), 9.78 (m, N***H***, 2H). ^{13}C NMR (50 MHz, [D6] DMSO): 10.62 (**C**H$_3$CH$_2$CH$_2$OOC–), 21.51 (CH$_3$**C**H$_2$CH$_2$OOC–), 25.68 (**C6**, **C6′**), 25.88 (**C4**), 31.8 (**C7**), 33.2 (C5, C5′), 34.44, 37.2 (**C3**), 57.4 (**C8**,), 67.6 (**C2**, CH$_3$CH$_2$**C**H$_2$OOC–), 170.7 (**C1**); IR (ATR): $\bar{\nu}$ = 2900–2500, 1733, 1448, 1215, 846. UV/Vis (DMSO): λ_{max} (ε_{max}/104 dm^3 mol^{-1} cm^{-1}): 269.11 nm (0.88). ESI/MS/TOF: positive mode *m/z* 453.37 [M–PtCl$_4$+H]$^+$, negative mode *m/z* 784.13 [M – H]$^-$, 785.19 [M]$^-$. Anal. Calcd for $C_{26}H_{48}O_4N_2Cl_4Pt$: C, 39.55; H, 6.13; N, 3.55, found: C, 40.04; H, 6.11; N, 3.66.

Elemental analyses were carried out with Elemental Vario EL III microanalyser (Elementar Analysensysteme GmbH, Hanau-Germany). Infrared spectra were recorded on a Nicolet 6700 FT-IR spectrometer using ATR technique (Thermo Electron Scientific Instruments Corp., Madison, WI). The NMR spectra were recorded on a Varian Gemini 200 instrument (Varian, Santa Clara, CA). Chemical shifts for ^{1}H and ^{13}C spectra were referenced to residual ^{1}H and ^{13}C presented in deuterated DMSO ([D6]DMSO). Mass spectra were carried out on an MS system consisted of 6210 Time-of-Flight LC/MS (G1969 A, Agilent Technologies, Santa Clara, CA) in DMSO/water solution, in both positive and negative ion mode. Electronic spectra were carried out on GBC UV–Visible Cintra 6 spectrometer (GBS, Melbourne, Australia), in DMSO, 0.2 mmol L^{-1} solution of complexes.



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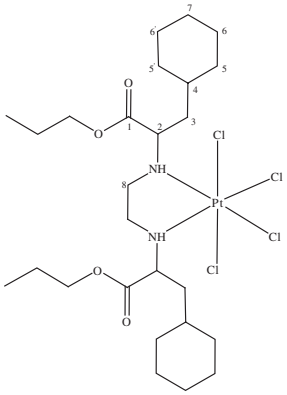

[Pt(L)Cl$_4$]

Melting points were determined on Electrothermal melting point apparatus (Keison Products, Chelmsford, Essex, UK).

3. Results

3.1. Effect of [Pt(L)Cl$_4$] on spleen cells (SPC) and lymph node cells (LNC) viability

In order to asses effect of [Pt(L)Cl$_4$] on immune cell viability, SPC and LNC isolated from rats and mice were stimulated with concanavalin A (ConA) for 24 h in the presence of various concentrations of the complex. In parallel, the cells were treated with L to determine relative contribution of the ligand to the effects of [Pt(L)Cl$_4$]. The cells were also treated with CP in order to compare the influence of [Pt(L)Cl$_4$] with the influence of this chemotherapeutic. SPC and LNC viability, determined by MTT assay, was significantly affected by 10 μM and 50 μM [Pt(L)Cl$_4$] and 50 μM L, both in rat and mouse cell cultures (Fig. 1). Also, [Pt(L)Cl$_4$] had stronger effect on SPC and LNC viability than CP (Fig. 1). Still, even in concentrations of 10 μM [Pt(L)Cl$_4$] and of 50 μM (L), their influence on SPC and LNC viability is bellow IC$_{50}$ value. Namely, [Pt(L)Cl$_4$] limited cell viability to 58.6%, 68.0%, 67.1% and 60.2% and L to 62.0%, 75.2%, 62.0% and 61.6% in mouse SPC, mouse LNC, rat SPC and rat LNC, respectively. Estimated IC50 values in μM for [Pt(L)Cl$_4$] are 53.0, 54.9, 57.4, 30.5 and for L are 66.1, 101.9, 67.9, 67.9 in mouse SPC, mouse LNC, rat SPC and rat LNC, respectively. Our previous report on the influence of [Pt(L)Cl$_4$] and L on tumor cell lines shows that IC$_{50}$ values vary from 2.2 μM to 3.1 μM for [Pt(L)Cl$_4$] and from 28.5 μM to 46.4 μM for L [11]. If we compare those results with above mentioned, newly gained data, it is clear that in the concentrations which are effective against transformed cells [Pt(L)Cl$_4$] and L exert limited effect on immune cell viability.

3.2. Effect of [Pt(L)Cl$_4$] on SNC and LNC generation of interferon (IFN)-γ and interleukin (IL)-17

Our further aim was to investigate influence of [Pt(L)Cl$_4$], L and CP on effector functions of immune cells. To this end, release of cytokines IFN-γ and IL-17 from SPC and LNC stimulated with ConA for 24 h in the presence of various concentrations of [Pt(L)Cl$_4$] or L or CP was determined. [Pt(L)Cl$_4$] potently reduced generation of IFN-γ (Fig. 2) and IL-17 (Fig. 3) in SPC and LNC of mouse and rat origin, while L and CP inhibitory effect on the cytokines' generation was almost exclusively statistically significant in 50 μM

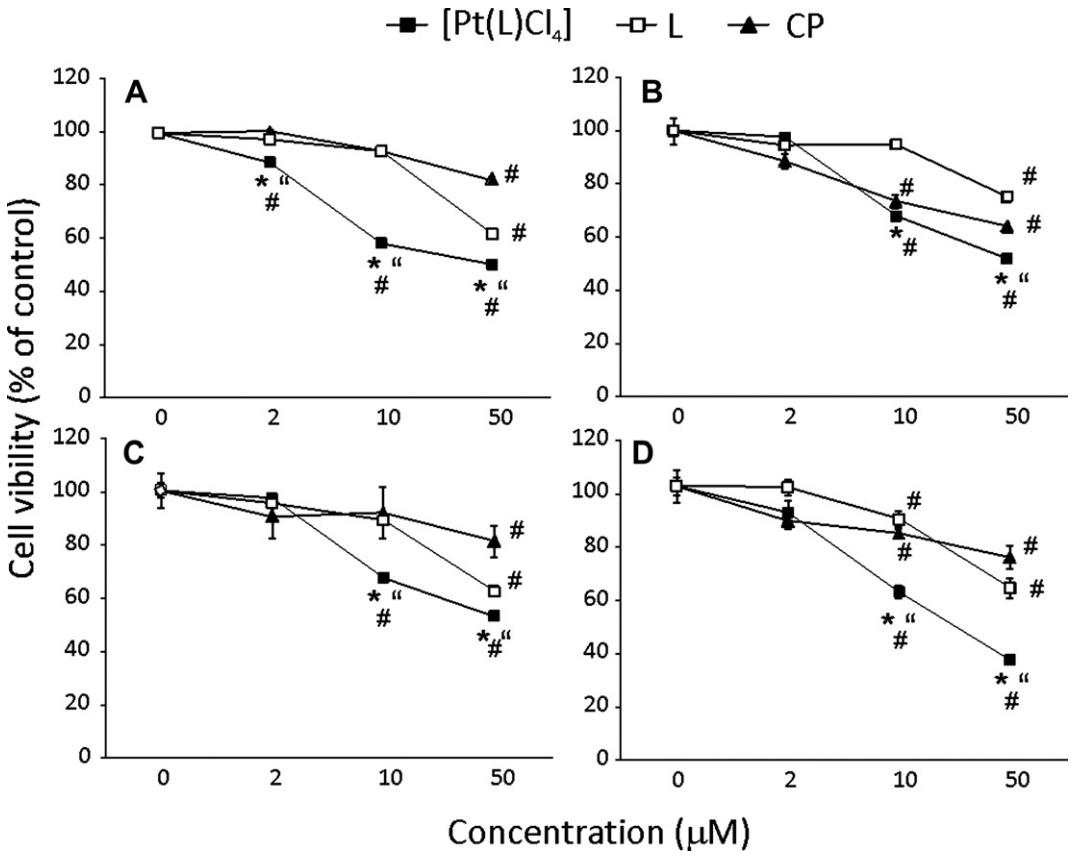

Fig. 1. Effect of [Pt(L)Cl4] on LNC and SPC viability. SPC (A, C) and LNC (B, D) isolated from mice (A, B) or rats (C, D) were stimulated with ConA in the presence of various concentrations of [Pt(L)Cl4] or L or CP for 24 h. Subsequently, MTT was performed and absorbance measured at 540 nm. Results presented as % of control values obtained in cultures untreated with any of the compounds (0) are mean ± SD of triplicate measurements from a representative of at least three independent experiments. #$p < 0.05$ represents statistical significance relative to untreated cultures (0), *$p < 0.05$ represents statistical significance between [Pt(L)Cl4]-treated and L-treated cultures, "$p < 0.05$ represents statistical significance between [Pt(L)Cl4]-treated and CP-treated cultures.

concentration (Figs. 2 and 3). Interestingly, [Pt(L)Cl_4] exerted stronger effect on IL-17 than on IFN-γ, as the complex significantly inhibited IL-17 synthesis even at 2 μM concentration. The effect of [Pt(L)Cl_4] and L on the cytokines' generation was, at least partly, mediated through inhibition of their gene expression. Relative IFN-γ gene expression of ConA-stimulated rat SPC was markedly down regulated by the influence of the agents after 24 h of cultivation (Fig. 4A). Similar inhibition was observed for IL-17 gene expression in the same cell cultures (Fig. 4B). Thus, although [Pt(L)Cl_4] did not potently affect SPC and LNC viability, it strongly inhibited their capacity to generate IFN-γ and IL-17.

3.3. *Effects of [Pt(L)Cl_4] on peritoneal cells (PC) viability and NO synthesis*

Further, the effect of [Pt(L)Cl_4], L and CP on PC was investigated. Mouse PC were stimulated with lypopolysaccharide (LPS)+IFN-γ and rat PC were stimulated with LPS in the presence of various concentrations of [Pt(L)Cl_4] or L or CP for 24 h. Viability of the cells was determined by crystal violet, and NO release was measured through detection of nitrite accumulation in cell culture supernatants. While the effect of [Pt(L)Cl_4], L and CP on PC viability was only marginal, [Pt(L)Cl_4], but not L, efficiently inhibited NO generation by these cells (Fig. 5). Also the influence of [Pt(L)Cl_4] on NO generation in PC was more profound than the effect of CP. Thus, [Pt(L)Cl_4] potently inhibited the synthesis of this effector molecule by immune cells.

4. Discussion

In this work we show that our novel Pt(IV) complex does not significantly affect cell viability of immune cells in the concentrations that are effective against transformed cells. Also, we present that it potently inhibits synthesis of IFN-γ, IL-17 and NO in immune cells.

CP is one of the most extensively used anticancer agents in the treatment of various malignancies, including cancers of the testes, ovary, bladder, head and neck, esophagus, stomach and lung, as well as lymphoma and osteosarcoma [2]. Still, severe toxicity, drug resistance and poor oral bioavailability represent major obstacles for its therapeutic use [1–3]. In attempts to overcome these CP weaknesses, the development of modified CP-based drugs has emerged. Numerous complexes of Pt with organic ligands have been synthesized, some of which have progressed to candidate cancer drugs, including carboplatin, oxaliplatin, satraplatin, picoplatin and triplatin [3]. As an example, satraplatin has a favorable toxicity profile and seems to be effective against a variety of malignancies, while the oral route of administration and the discontinuous application make it very convenient for use in patients [12]. However, despite encouraging preclinical studies, results of clinical trials of satraplatin and other Pt-based agents were below expectations [3,12]. Therefore, there is an open field for investigation of newly synthetized Pt-complexes which could have better properties than CP. We have recently reported that [Pt(L)Cl_4] and its ligand L are potent against transformed cell lines *in vitro* [11]. Importantly, IC_{50} values for [Pt(L)Cl_4] were at least three folds

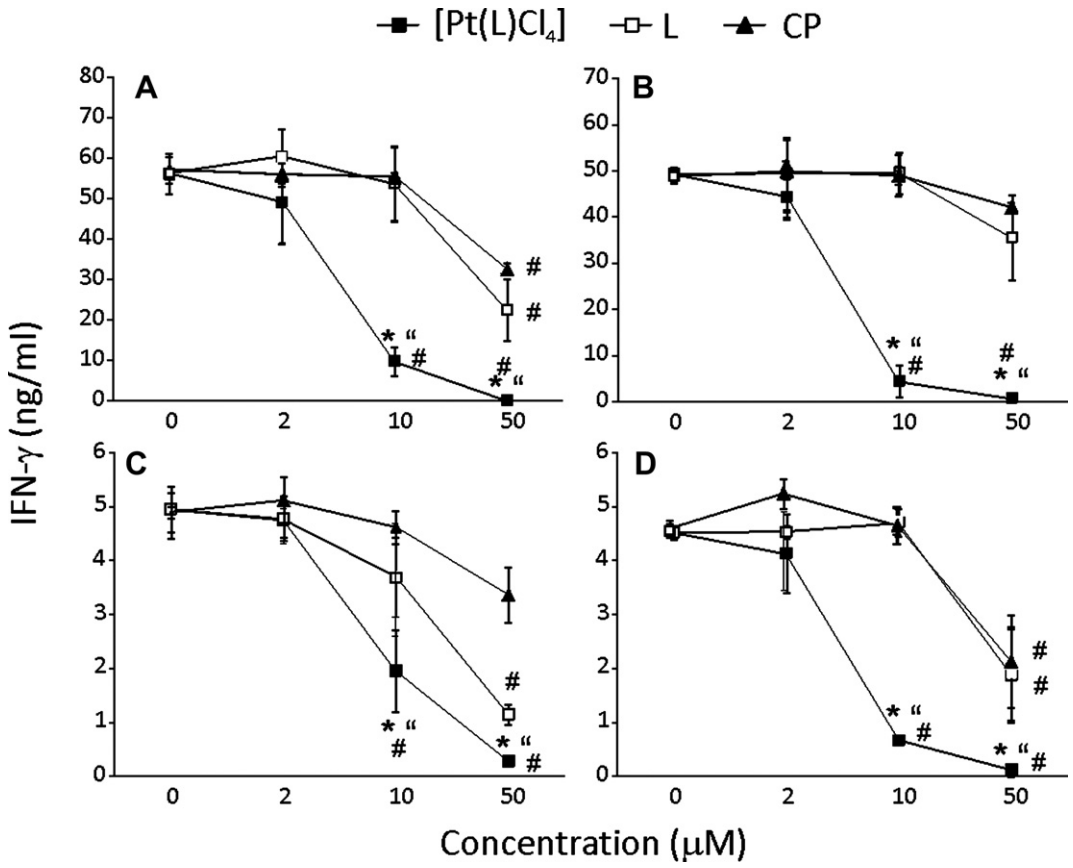

Fig. 2. Effect of [Pt(L)Cl4] on IFN-γ release from LNC and SPC. SPC (A, C) and LNC (B, D) isolated from mice (A, B) or rats (C, D) were stimulated with ConA in the presence of various concentrations of [Pt(L)Cl_4] or L for 24 h. Subsequently, cell-free supernatants were collected, ELISA for IFN-γ was performed and concentrations of the cytokine determined. Results are presented as mean ± SD from three independent experiments. $^{\#}p < 0.05$ represents statistical significance relative to untreated cultures (0),$^{*}p < 0.05$ represents statistical significance between [Pt(L)Cl_4]-treated and L-treated cultures, and $^{“}p < 0.05$ represents statistical significance between [Pt(L)Cl_4]-treated and CP-treated cultures.

less than corresponding IC_{50} values of CP. Here, we were interested in investigating toxicities of the agents. As myelosuppression and leucopenia are among the listed side effects of CP [2,13], we investigated influence of [Pt(L)Cl_4] and L on the cells of hematologic origin, i.e. LNC, SPC and PC. In the concentrations that were efficient against transformed cell lines, [Pt(L)Cl_4] and L had only limited effect on viability of immune cells, thus implying that the novel Pt complex, as well as its ligand, might be devoid of myelosupressive and leucopenic effects.

In addition, we were interested in investigating immune-related effects of the agents, as it has recently been shown that CP-induced nephrotoxicity in mice is mediated by immune cells, CD4 T cells in particular [14]. Therefore, we examined [Pt(L)Cl_4] and L influence on classical proinflammatory CD4 T cell cytokines IFN-γ and IL-17, which are produced by T helper (Th)1 and Th17 cells, respectively [15]. Generation of these cytokines was downregulated in LNC and SPC, and both [Pt(L)Cl_4] and L inhibited their mRNA expression. In contrast, CP was previously shown to stimulate IFN-γ mRNA expression in human peripheral blood cells [16]. In our research, CP inhibited generation of IFN-γ and IL-17 in LNC and SPC, but less efficiently then [Pt(L)Cl_4]. As it has recently been reported that $CD4^{+}CD25^{+}$ regulatory T cells (Treg) attenuate CP-induced nephrotoxicity in mice [17], our result that [Pt(L)Cl_4] limits IFN-γ and IL-17 production might be of special interest. Importantly, Treg counteract Th17 activity and inhibit generation of IL-17 [15], and it was reported that in various diseases affecting urinary functions, Treg/Th17 balance is shifted toward Th17 cells [18–20]. Thus, our finding that [Pt(L)Cl_4] inhibits IL-17 generation implies that this agent should have less, if any, nephrotoxic effects than CP. This assumption should be examined as a part of a detailed toxicological study of the agent. Still, it has to be noted that both IFN-γ and IL-17 are important stimulators of immune response, including anti-tumor immunity. This is an especially important view regarding IFN-γ, as it has convincingly been shown that this cytokine potentiates immunogenicity of tumor cells, migration of immune cells into transformed tissue and generation of tumoricidal effector molecules within tumors [22]. Therefore, inhibitory effects of the novel Pt(IV) compound on IFN-γ might also be counterproductive. On the other hand, there have been contradictions regarding the effect of IL-17 on tumors, ranging from protumor to antitumor actions of this cytokine depending on the phase of tumor development and the type of tumor examined [21]. Therefore, it seems reasonable that downregulation of IL-17 in patients could be beneficial, at least at some therapy phases.

Furthermore, [Pt(L)Cl_4] potently inhibits NO generation by rat peritoneal macrophages. In contrast, previous reports show that CP potentiates NO release from macrophages, and this effect of CP has been associated with its antitumor activity [22,23], but also with induction of apoptosis in macrophages [24]. Still, CP did not potentiate, but rather inhibited NO release from PC in our experiments, although less efficiently then [Pt(L)Cl_4]. This discrepancy between previous reports and our data might be explained by the use of different cells, as we used peritoneal cells and Sodhi and Suresh used bone marrow derived macrophages [22]. Also, another difference between our work and that of Chauhan and colleagues is that we neither co-cultivated our macrophages with tumor cells

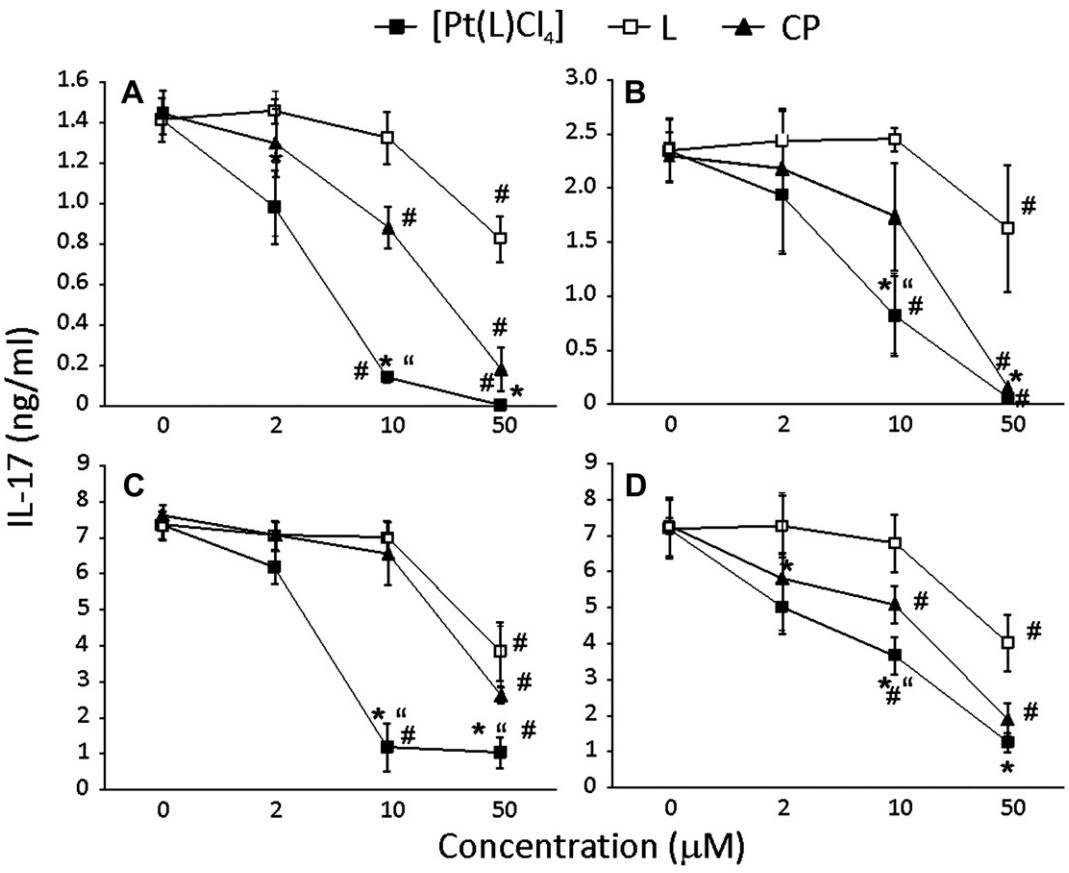

Fig. 3. Effect of [Pt(L)Cl4] on IL-17 release from LNC and SPC. SPC (A, C) and LNC (B, D) isolated from mice (A, B) or rats (C, D) were stimulated with ConA in the presence of various concentrations of [Pt(L)Cl_4] or L or CP for 24 h. Subsequently, cell-free supernatants were collected, ELISA for IL-17 was performed and concentrations of the cytokine determined. Results are presented as mean ± SD from three independent experiments. $^{\#}p < 0.05$ represents statistical significance relative to untreated cultures (0),$^{*}p < 0.05$ represents statistical significance between [Pt(L)Cl_4]-treated and L-treated cultures, and $^{“}p < 0.05$ represents statistical significance between [Pt(L)Cl_4]-treated and CP-treated cultures.

nor pretreat them with CP [23]. NO is typically associated with killing of NO-sensitive tumors by macrophages [25]. But in some cases, it can also promote tumor growth and survival through mimicking hypoxia, as well as by inducing tumor cell apoptosis, which in turn inhibits antitumor activity of macrophages [25]. Thus, our finding that [Pt(L)Cl_4] inhibits NO in macrophages does not necessarily represent a shortcoming for its antitumor properties.

Regarding potential immunomodulatory properties of the agent, its inhibitory influence on IFN-γ and IL-17 release from T cells, as well as on NO generation in macrophages might be a beneficial property in various inflammatory disorders. Although these effector molecules are efficient in the immune response against various pathogens, their excessive and uncontrolled production has reportedly been associated with pathogenesis of various inflammatory disorders, including autoimmune diseases [26–28]. Therefore, through inhibition of the synthesis of these molecules, the novel Pt complex might contribute to the limitation of chronic inflammation which is the major culprit in various pathologies, including cardiovascular, metabolic, neurodegenerative,

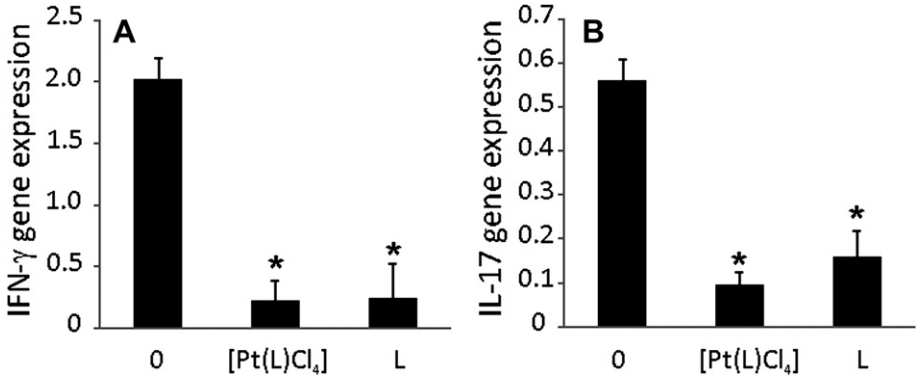

Fig. 4. Effect of [Pt(L)Cl4] on IFN-γ and IL-17 gene expression in SPC. SPC isolated from rats were stimulated with ConA in the presence of [Pt(L)Cl4] (10 μM) or L (50 μM) for 24 h. Subsequently, RNA was isolated from cultures and RT-PCR was performed. Results presented as gene expression of IFN-γ (A) and IL-17 (B) relative to actin, are mean ± SD from three independent experiments. $^{*}p < 0.05$ represents statistical significance relative to control (0) cultures.

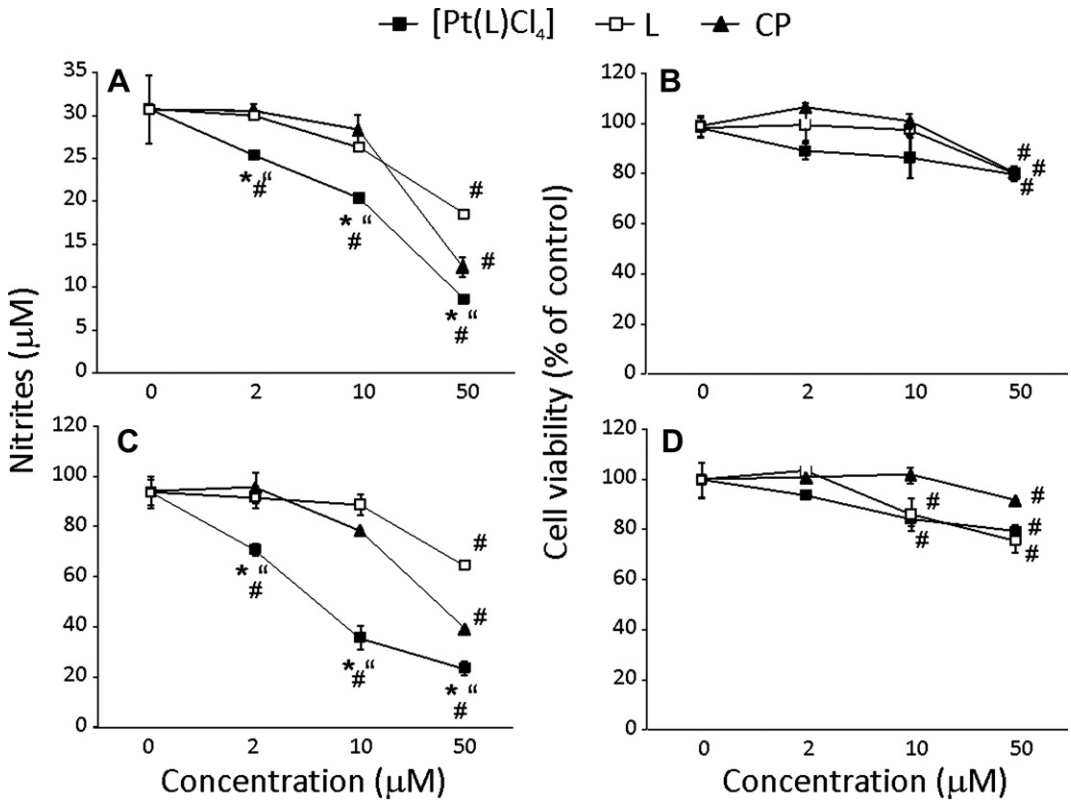

Fig. 5. Effect of [Pt(L)Cl4] on PC viability and NO generation. PC isolated from mice (A, B) were stimulated with LPS+IFN-γ and PC isolated from rats (C, D) were stimulated with LPS in the presence of various concentrations of [Pt(L)Cl4] or L or CP for 24 h. Subsequently, cell-free supernatants were collected and concentration of nitrites determined. Also, CV was performed and absorbance measured at 540 nm. Results of CV are presented as % of control values obtained in cultures untreated with any of the compounds (0). Results are presented as mean ± SD of triplicate measurements from a representative of at least three independent experiments. #$p < 0.05$ represents statistical significance relative to untreated cultures (0),*$p < 0.05$ represents statistical significance between [Pt(L)Cl4]-treated and L-treated cultures, and "$p < 0.05$ represents statistical significance between [Pt(L)Cl4]-treated and CP-treated cultures.

autoimmune and allergic disorders [29–31]. Furthermore, chronic inflammation has been associated with cancer pathology [32]. Inflammatory mediators have been considered to influence various important steps in tumor development, including initial transformation of a normal cell, tumor cell survival and proliferation, induction of chemoresistance and radioresistance, as well as invasion and angiogenesis [32]. Thus, immunomodulatory properties of [PT(L)Cl_4] might contribute to the overall antitumor properties of the complex.

5. Conclusion

Taken together, results presented in this paper suggest that [Pt(L)Cl_4], a novel Pt-based complex with potent anticancer activities does not exert general immunosuppressive effect, but rather has immunomodulatory activity which might contribute to the overall therapeutic properties of the complex. These results invoke investigation of [Pt(L)Cl_4] in animal models of cancer, as the next step in the investigation of its chemotherapeutic quality, and also in animal models of chronic inflammatory disorders.

6. Experimental protocols

6.1. Cells and cell cultures

Experimental animals (C57BL/6 mice and Dark Agouti rats) were obtained from the Animal House Facility of the Institute for Biological Research "Siniša Stanković", Belgrade. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the institute. SPC were isolated from spleens and LNC were obtained from cervical lymph nodes. The organs were mechanically disrupted, passed through 40 μm nylon mesh filter and the resulting suspension was collected by centrifugation. Red blood cells from single cell suspensions obtained from spleens were lysed using RBC Lysis Buffer (eBioscience, San Diego, CA). SPC and LNC were seeded at 5 x 10^6/ml/well of 24-well plates. PC were obtained by a lavage of peritoneal cavity with ice-cold PBS. PC (2 x 10^6/ml/well) were plated into 24-well plates, left at 37 °C for 2 h and then cell culture supernatant containing non-adherent cells was removed, leaving adherent macrophages in cultures. SPC and LNC were stimulated with 1 μg/ml ConA (Sigma–Aldrich, St. Louis, MO) and rat PC with 10 ng/ml LPS (E. coli 055:B5, Sigma–Aldrich) and mouse PC with 10 ng/ml LPS and 10 ng/ml recombinant murine IFN-γ (Sigma–Aldrich).

6.2. Cell viability assays

In order to assess the viability of SPC and LNC we used the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan. At the end of appropriate treatments, SPC and LNC were collected in tubes, spun down, supernatants removed and the cell pellets dissolved in 0.5 μg/ml MTT (Sigma–Aldrich) solution. Incubation with MTT lasted for 30 min at 37 °C and cells were centrifuged once more. DMSO was added to the pellets to dissolve the formazan crystals. In order to determine viability of PC, crystal violet (CV) staining was

performed. Cell culture supernatants were collected and the cells fixed with methanol (10 min at room temperature). Subsequently, cells were stained with CV solution (2% in PBS v/v, 10 min, room temperature) and then thoroughly washed with tap water. Finally, crystal violet dye remaining in the cells was dissolved in 33% acetic acid. The absorbances in MTT assay and CV assay were measured at 570 nm, with a correction at 690 nm, using an automated microplate reader (LKB 5060-006, LKB, Vienna, Austria).

6.3. Measurement of NO release

Nitrite accumulation, as an indirect measure of NO release, was determined in cell culture supernatants using the Griess reaction. In brief, triplicate aliquots of cell-free supernatants were mixed with an equal volume of Griess reagent, a 1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride (Sigma–Aldrich) and 1% sulphanilamide (Sigma–Aldrich) in 5% H_3PO_4. The absorbance at 570 nm was determined in a microplate reader (LKB 5060-006; LKB) and compared to a standard curve for $NaNO_2$.

6.4. ELISA

Cytokine concentration in cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Rochild, Denmark) and anti-cytokine paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine/rat IL-17 and murine IFN-γ (eBioscience, San Diego, CA) and rat IFN-γ (R&D, Minneapolis, MN). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

6.5. Reverse transcription–real time Polymerase chain reaction

In order to determine cytokine gene expression real time RT-PCR was performed. First, total RNA was isolated from SPC (5×10^6 cells) using a mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) and reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase, according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the recommendations of the manufacturer in a total volume of 20 μl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermocycler conditions comprised an initial step at 50 °C for 5 min, followed by a step at 95 °C for 10 min and a subsequent 2-step PCR program at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. The PCR primers (Metabion) were as follows: β-actin forward primer 5′-GCT TCT TTG CAG CTC CTT CGT-3′; β- actin reverse primer 5′-CCA GCG CAG CGA TAT CG-3′; IFN-γ forward primer 5′-TGG CAT AGA TGT GGA AGA AAA GAG-3′; IFN-γ- reverse primer 5′-TGC AGG ATT TTC ATG TCA CCA T-3′; IL-17 forward primer 5′-ATC AGG ACG CGC AAA CAT G-3′; IL-17 reverse primer 5′-TGA TCG CTG CTG CCT TCA C-3′. Accumulation of PCR products was detected in real time and the results analyzed with 7500 System Software (Applied Biosystems) and presented as 2^{-dCt}, dCt being the difference between Ct values of specific gene and the endogenous control (β-actin).

6.6. Statistical analysis

A Student's *t* test was performed for statistical analysis. A *p* value less than 0.05 was considered statistically significant.

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