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Cytotoxicity and antiviral activity of palladium(II) and platinum(II) complexes with 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone

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Abstract: Metal coordination compounds have an important role in the development of novel drugs. Using the resazurin microtitration assay we assessed the cytotoxicity and antiviral activity of the ligand 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone and its Pd(II) and Pt(II) complexes. Cytotoxicity was tested in A549 human lung adenocarcinoma epithelial cells. We observed that the ligand displayed a more pronounced cytotoxic activity than the platinum-based drug, carboplatin. Morphological evaluation of A549 cells treated with the ligand by acridine orange and ethidium bromide double staining revealed the presence of signs of apoptosis. Antiviral activity against poliovirus type 1 was assessed by examination of the cytopathic effect (CPE) in Hep-2 cells. Cells that were exposed to the 19 μM ligand before infection displayed a maximal significant reduction (by 24.42 ± 1.49%) of the CPE. This was likely due to the inhibition of virus receptors and prevention of viral adsorption. Treatment with 17 μM Pt(II) complex after viral infection caused a maximal significant reduction (by 30.52 ± 3.12%) of the CPE, presumably through an effect on viral replication. The results indicate that the ligand should be viewed as a potential anticancer agent. The ligand and the Pt(II) complex show promising results for further investigation of antiviral activity.

Key words: Cytotoxicity, antiviral activity, 2-(diphenylphosphino)benzaldehyde hydrazone ligands, cancer cell lines, apoptosis

1. Introduction

The clinical use of metal coordination compounds has improved considerably in recent years in clinical therapy. Platinum-based drugs (cisplatin, carboplatin, and oxaliplatin) have a wide use in the treatment of human malignancies, such as ovarian, testicular, head and neck, and lung cancers. Considerable effort has been devoted to the development of new anticancer metal coordination compounds that impede the development of resistance and produce fewer side effects during chemotherapy (Frezza et al., 2010).

Uncovering the anticancer properties of phosphines has led to the development and biological evaluation of numerous metal complexes with phosphine ligands (Starosta et al., 2011; Galassi et al., 2012; Milenković et al., 2013a, 2013b). Metal complexes with 2-(diphenylphosphino) benzaldehyde (dpba) hydrazone ligands also have improved cytotoxic activity (Mališević et al, 2006). In our previous work we investigated the biological activity of the novel synthesized ligand 2-(diphenylphosphino) benzaldehyde 1-adamantoylhydrazone and its Pd(II) and Pt(II) complexes (Figure 1) in human larynx carcinoma Hep-2 cells and healthy human lung MRC-5 cells (Đorđević et al., 2014). The antiviral activities of Pt(II) and Pd(II) complexes with 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone ligand have not been studied.

Several studies have shown that palladium complexes have cytotoxic activities similar to standard platinum-based drugs (Gao et al., 2010). Some palladium complexes have better growth-inhibition effects on human nonsmall-cell lung cancer in vitro than cisplatin (Ulukaya et al., 2011).

Ligand 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone and its Pd(II) and Pt(II) complexes contain an adamantyl group, which changes the therapeutic profile of the pharmacologically active molecules (Liu et al., 2011). Compounds with the adamantyl group are widely used for prophylaxis and treatment of influenza infections (Monto, 2003) and also possess antiviral potency against viruses of the family *Picornaviridae* (De

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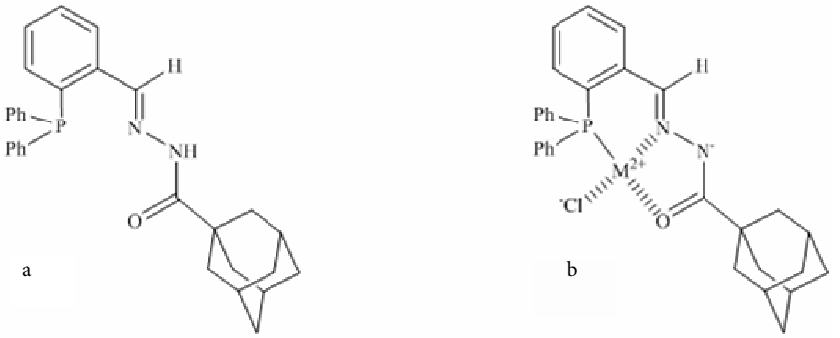

Figure 1. Chemical structures of a) ligand and b) palladium(II) and platinum(II) complexes (M = Pd, Pt).

Palma et al., 2008). Poliovirus, a member of the genus *Enterovirus* (family *Picornaviridae*), is a causative agent of paralytic poliomyelitis. The Hep-2 cell line is a suitable host cell model with an elevated sensitivity to poliovirus infection. Hep-2 cells provide a high yield of poliovirus and exhibit a visible and reliable level of cytopathic effect (CPE) (Johnston and Siegel, 1990; WHO, 1997).

In this study we examined the cytotoxicity of ligand 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone and its Pt(II) and Pd(II) complexes in A549 human lung adenocarcinoma cells, and their antiviral activity against poliovirus type 1 in Hep-2 cells using the resazurin microtitration assay (UptiBlue assay).

2. Materials and methods

2.1. Reagents and chemicals

Minimal essential medium (MEM, GIBCO), penicillin-streptomycin (Pen Strep, 10,000 U/mL penicillin, 10,000 µg/mL streptomycin, GIBCO), nonessential amino acids (MEM NEAA, GIBCO), L-glutamine (2 mM, GIBCO), fetal bovine serum (FBS, GIBCO), nutrient medium (MEM, 1% Pen Strep, 1% MEM NEAA, 1% L-glutamine, supplemented with FBS), trypsin-EDTA (0.05%, GIBCO), UptiBlue (Uptima, Interchim), acridine orange (10 mg/mL, Sigma), ethidium bromide (10 mg/mL, Sigma), Dulbecco's phosphate-buffered saline (DPBS, GIBCO), dimethyl sulfoxide (DMSO, Sigma-Aldrich), cisplatin (0.5 mg/mL, Cisplatin, Medac), carboplatin (10 mg/mL, Carboplatin-Teva, Teva), oxaliplatin (5 mg/mL, Eloxatin, Sanofi-Aventis), interferon α-2a (3×10^6 IU/0.5 mL, Roferon-A, Roche), poliovirus reference standard (2nd International Reference Reagent 2004 for Live Attenuated Poliovirus (Sabin), National Institute for Biological Standards and Control, code: 02/306), and antipoliovirus sera (3rd International Standard Anti-Poliovirus Serum Types 1, 2, and 3, National Institute for Biological Standards and Control, code: 82/585) were used.

2.2. Test compounds

2-(Diphenylphosphino)benzaldehyde 1-adamantoylhydrazone (HL) (43.1 mM, referred to as compound '1'); Pd(II) complex with 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone, [Pd(L)Cl], (34.3 mM, compound '2'); and Pt(II) complex with 2-(diphenylphosphino) benzaldehyde 1-adamantoylhyd-razone, [Pt(L)Cl], (19.8 mM, compound '3') were used at the indicated concentrations.

2.3. Virus

As a starting virus suspension, we used the poliovirus reference standard, which contains a mixture of poliovirus types 1, 2, and 3. Antipoliovirus sera types 2 and 3 were added to the poliovirus reference standard. Poliovirus types 2 and 3 were neutralized at 37 °C for 3 h. After neutralization of poliovirus types 2 and 3, the content of poliovirus type 1 was determined by the microtitration assay in Hep-2 cell culture, using the Kärber formula. The results were expressed as a tissue culture infection dose per milliliter ($TCID_{50}$/mL) (WHO, 1997). The stock virus suspension was aliquoted and stored at –20 °C until use. Dilution of the virus suspension was prepared in nutrient medium supplemented with 2% FBS.

2.4. Cell cultures

A549 human lung adenocarcinoma epithelial cells and Hep-2 human larynx carcinoma cells were cultivated in a nutrient medium supplemented with 5%–10% FBS at 37 °C and 5% CO_2. The confluent monolayer was trypsinized with 0.05% trypsin-EDTA. The cells were resuspended in a nutrient medium supplemented with 10% FBS and placed into cell culture 96-well microtiter plates (Nunc), at a concentration of 1×10^5 cells/mL. The microtiter plates were incubated for 24 h at 37 °C and 5% CO_2 humidified air. The quality of the cell monolayer was confirmed with an inverted microscope (Freshney, 2005).

2.5. Cytotoxicity assay in A549 cells

The cytotoxic potential of test compounds 1, 2, and 3 was assayed in the A549 lung adenocarcinoma epithelial cell line, using UptiBlue (Vega-Avila and Pugsley, 2011).

The A549 cell line is chosen for the cytotoxicity examination due to a high potential for resistance development to cisplatin (Cetintas et al., 2012). The cytotoxic drugs cisplatin, carboplatin, and oxaliplatin served as positive controls. Two-fold serial dilutions were prepared in nutrient medium supplemented with 10% FBS, starting from 336.5 μM, 268 μM, and 155.2 μM for compounds 1, 2, and 3, respectively, and from 416 μM, 672 μM, and 628 μM for cisplatin, carboplatin, and oxaliplatin, respectively. Diluted compounds were added to microtiter plates with an A549 cell monolayer. The cells were incubated for 48 h at 37 °C and 5% CO_2. The following controls were included: a cell control consisting of untreated cells; a control of the solvent, which consisted of cells in two-fold serial dilutions of DMSO, starting from 0.78%, that corresponded to the test compounds; and nutrient medium supplemented with 10% FBS, which served as the blank. Changes in cell morphology were observed through an inverted phase contrast microscope. After the treatment, the plates were subjected to the UptiBlue assay.

UptiBlue is resazurin-based viable stain and it is used as an oxidation-reduction indicator that undergoes colorimetric change in response to cellular metabolic reduction. The amount of resazurin conversion into resorufin is linearly related to the number of viable cells and it can be measured spectrophotometrically.

In the UptiBlue assay the medium was removed after the respective treatments. After the cell monolayers were rinsed with DPBS, 10% of UptiBlue solution diluted in nutrient medium supplemented with 10% FBS was added. The plates were incubated for an additional 3 h at 37 °C and 5% CO_2. Absorbance was recorded using a Thermo Labsystems Multiscan Acsent plate reader at 540 nm and 600 nm.

The percentages of cell survival were calculated according to the instructions for UptiBlue. IC_{50} values in the cytotoxicity assay were defined as the concentrations of the compounds that inhibited 50% of cell growth as compared to the positive growth control. IC_{50} values were determined from the cell growth diagrams.

2.6. Acridine orange and ethidium bromide double staining

Morphological analysis of apoptosis by double staining with acridine orange and ethidium bromide (AO/EB) was used to distinguish live nonapoptotic, apoptotic, and necrotic cells, based on membrane integrity and chromatin condensation (Squier and Cohen, 2001). AO is taken up by both viable and nonviable cells and emits green fluorescence if bound to nuclear DNA and red fluorescence if bound to RNA, mitochondrial DNA, or secretion granules (lysosomes). Ethidium bromide permeates only nonviable cells with damaged membranes and emits red fluorescence by intercalation into DNA.

The A549 cells were treated with the IC_{50} concentration of ligand for 48 h. After the treatment, A549 cells were washed with DPBS and trypsinized. The cells were stained with 2 μL of AO/EB solution (100 μg/mL AO and 100 μg/mL EB) and observed under a fluorescence microscope (Leica) at 400× magnification within 30 min before the fluorescence color started to fade. According to the fluorescence emission and the morphological aspect of chromatin condensation of stained nuclei, the cells were distinguished as described by Baskić et al. (2006).

2.7. Antiviral activity against poliovirus type 1 in Hep-2 cells

Antiviral activity was expressed as the ability of the agents to reduce poliovirus type 1 induction of CPE in Hep-2 cells as observed by the UptiBlue assay. The antiviral activity assay included two types of treatments with the ligand and the Pd(II) and Pt(II) complexes that are referred to as preinfection and postinfection treatments. Two-fold serial dilutions of compounds 1, 2, and 3 were prepared in nutrient medium supplemented with 2% FBS, starting from 19 μM, 8.24 μM, and 17 μM, respectively, based on the reported cytotoxicity results obtained previously in the Hep-2 cell line (Đorđević et al., 2014). Cells treated with interferon α-2a served as a positive control of antiviral activity (Cantell and Strander, 1966). It was previously established that interferon α-2a at concentrations of up to 1000 IU/mL are not cytotoxic. The highest applied concentration of interferon α-2a was 400 IU/mL. The test included a cell control (untreated cells), a solvent control (cells with two-fold serial dilutions of DMSO, starting from 0.1%, which corresponded to the test compounds), and a control of viral infectivity (cells incubated with the virus suspension); nutrient medium supplemented with 2% FBS provided the blank. At the end of the antiviral assay, 100% CPE was confirmed in the control for viral infectivity with an inverted phase contrast microscope. After the treatment, the plates were subjected to the UptiBlue assay.

EC_{50} values in the antiviral activity assay were defined as the concentrations of the compounds that inhibited 50% viral activity as compared to the positive growth control. EC_{50} values were read from the viral growth inhibition diagrams (CPE reduction diagrams). Antiviral activity was quantitatively expressed as the therapeutic index (TI), defined as the relative effectiveness of the test compound in inhibiting viral activity as compared to its effectiveness in inducing cell death (IC_{50} / EC_{50}) (FDA, 2006). When it was not possible to calculate the TI values, the results were expressed as percentages of CPE reduction.

2.7.1. Preinfection treatment assay

Hep-2 cell monolayers incubated with test compounds 1, 2, and 3 and the corresponding controls were maintained for 5 h at 37 °C and 5% CO_2. After incubation, the compounds

were removed, and the cell monolayers were rinsed with DPBS and infected with a suspension of poliovirus type 1 (100 $TCID_{50}$/mL). After a 7-day incubation, the plates were subjected to the UptiBlue assay.

2.7.2. Postinfection treatment assay

Hep-2 cell monolayers were incubated with a poliovirus type 1 suspension (100 $TCID_{50}$/mL) for 90 min at 37 °C and 5% CO_2. After incubation, the viral suspension was removed and the cell monolayers were rinsed with DPBS. Compounds 1, 2, and 3 and the corresponding controls were applied to Hep-2 cell monolayers. After a 7-day incubation, the plates were subjected to the UptiBlue assay.

2.8. Statistical analysis

The validity of the assay and results of poliovirus type 1 standardization were statistically interpreted using CombiStats (EDQM, 2009) software for statistical analysis of biological dilution assays. Calculations were done according to Chapter 5.3 of the European Pharmacopoeia (Council of Europe, 2013). Results obtained in the cytotoxicity and antiviral activity assays were expressed as mean values obtained from three independent experiments performed at least in triplicate. The data are presented as mean values ± standard error (SE). Student's t-test was used to evaluate the difference between the test data and control. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity in A549 cells

The results of the cytotoxicity in A549 cells showed that cisplatin and oxaliplatin have similar cytotoxic activities, with IC_{50} values at concentrations of 40 ± 1.4 µM and 42.6 ± 2.5 µM, respectively (Figure 2A). Carboplatin showed a lower cytotoxic capacity for inducing cell growth inhibition (IC_{50} = 256.1 ± 14.0 µM). The obtained IC_{50} value for the ligand was 165.9 ± 17.6 µM. Pd(II) and Pt(II) complexes did not exhibit cytotoxic activities in A549 cells (Figure 2B).

3.2. Morphological changes in A549 cells

The morphological changes in A549 cells were observed using inverted phase contrast (Olympus) and fluorescence (Leica) microscopes. After 48 h of incubation, most of the control A549 cells exhibited typical cell morphology: the cells were polygonal or slightly prolate and adherent to the tissue culture dishes (Figures 3A and 3B). Examination of the morphology of A549 cells treated with ligand at a concentration of 168.3 µM after 24 h (Figures 3C and 3D) and 48 h (Figures 3E and 3F) revealed different degrees of morphological changes, including loss of cell adhesion, membrane shrinkage, membrane blebbing, condensation of the cytoplasm and vacuolization, nuclear fragmentation, formation of apoptotic bodies and echinoid spikes, and cell fragmentation.

After AO/EB double staining, the control A549 cells showed typical morphology of live cells with bright green uniform nuclei (Figures 4A and 4B). After 48 h of treatment, most of the cells were in the early stage of apoptosis. The early apoptotic cells showed green nuclei and perinuclear chromatin condensation was visible as bright green patches (Figure 4C). Only a small fraction of the cell population was in the late apoptotic stage. The late apoptotic cells were identified by orange to red nuclei with condensed or fragmented chromatin (as a consequence of EB penetration through a damaged cell membrane) (Figure 4D).

3.3. Antiviral activity against poliovirus type 1 in Hep-2 cells

Interferon α-2a showed significant antiviral activity against poliovirus type 1 in both preinfection and postinfection

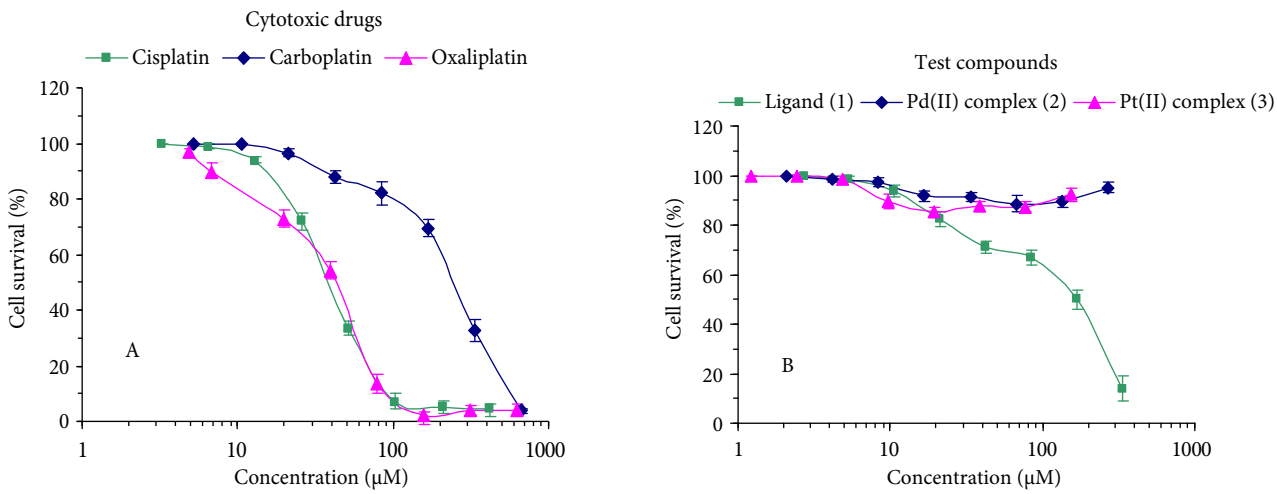

Figure 2. Cytotoxic effect of A) cisplatin, carboplatin, and oxaliplatin, and B) test compounds 1, 2, and 3 in A549 cells.

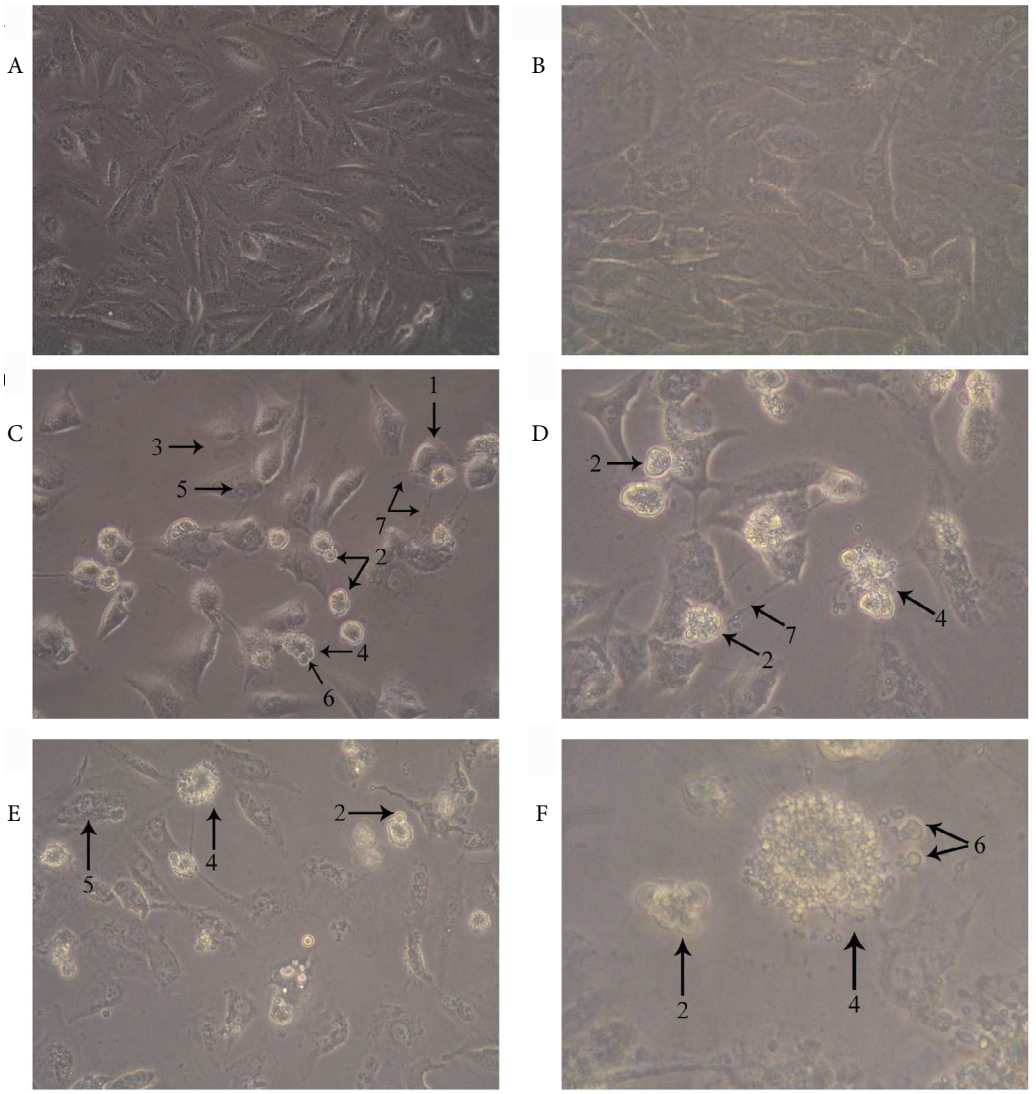

Figure 3. Representative photomicrographs showing the morphological changes of A549 cells after treatment with ligand, observed through an inverted phase contrast microscope (A, C, E: 200× magnification; B, D, F: 400× magnification). A, B – control A549 cells; C, D – 24 h of treatment; E, F – 48 of h treatment. Arrows point to (1) cell rounding, (2) membrane blebbing, (3) cell shrinkage, (4) cell fragmentation, (5) nuclear fragmentation, (6) apoptotic bodies, and (7) echinoid spikes.

treatments (Figure 5). In the preinfection treatment with interferon α-2a, the EC_{50} value was 32.5 ± 3.7 IU/mL, with a therapeutic index (TI) of greater than 30.8. In the postinfection treatment with interferon α-2a, the EC_{50} value was 51 ± 1.16 IU/mL, and the TI was greater than 19.6. The Pd(II) complex did not display an antiviral effect in either the preinfection or postinfection treatments, as the CPE remained at 100% for all of the tested concentrations (data not shown).

3.3.1. Preinfection treatment assay

The results of the preinfection treatment with the ligand and Pt(II) complex are presented in Figure 6. For the ligand, significant inhibition of the CPE was detected at 2.38 μM and above, with the highest effect observed at 19 μM (24.42 ± 1.49% of CPE reduction). For the Pt(II) complex, a significant inhibition of the CPE was detected at 4.25 μM and above, with the highest effect observed at 17 μM (11.14 ± 1.50% of CPE reduction). From these results it is evident that in the preinfection treatment the ligand showed a higher antiviral potential than the Pt(II) complex.

3.3.2. Postinfection treatment assay

The results of the postinfection treatment are presented in Figure 7. For the ligand, a significant inhibition of the CPE

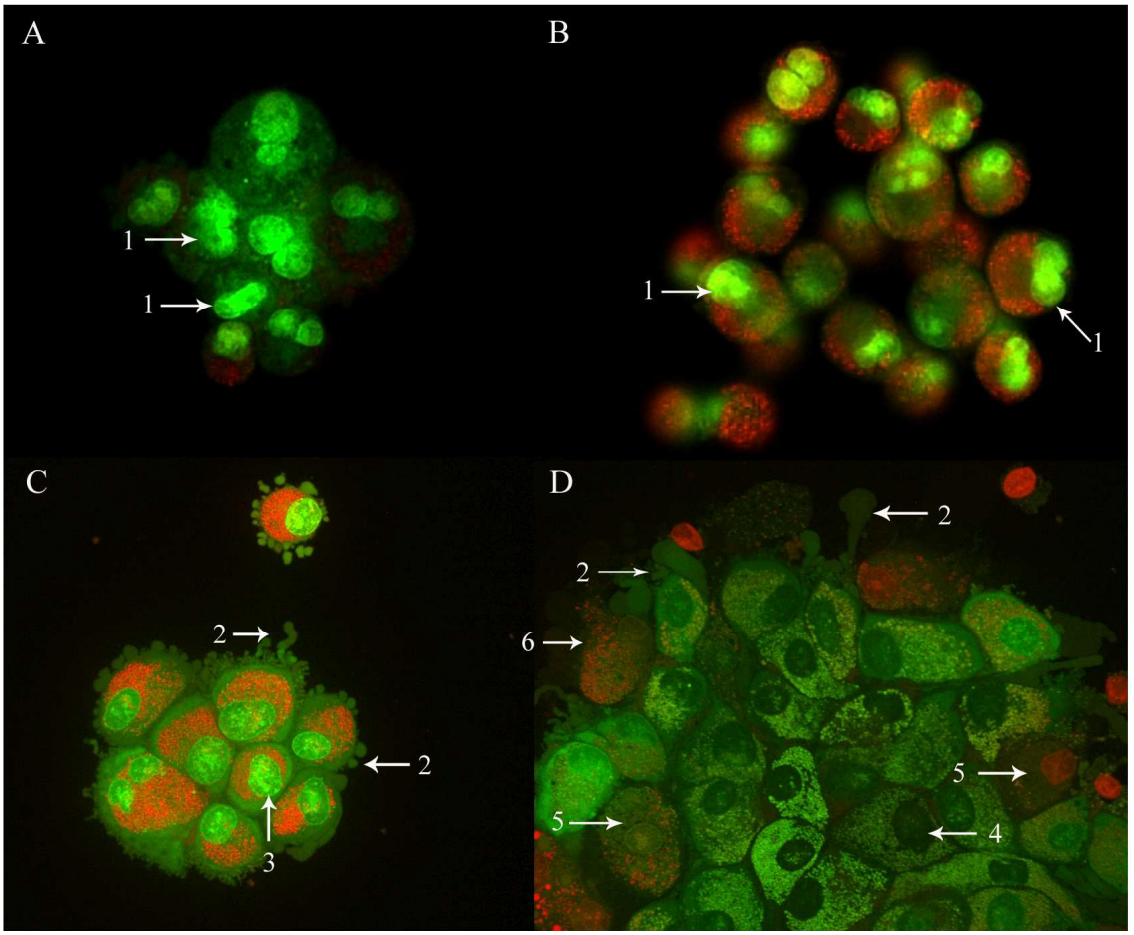

Figure 4. Representative photomicrographs showing the morphological changes of AO/EB-stained A549 cells after treatment with ligand, observed through a fluorescence microscope (400× magnification). A, B – control A549 cells; C, D – 48 h of treatment. Arrows point to (1) live nonapoptotic cells, (2) membrane blebbing, (3) chromatin condensation, (4) early apoptotic cell, (5) late apoptotic cell, and (6) cell fragmentation.

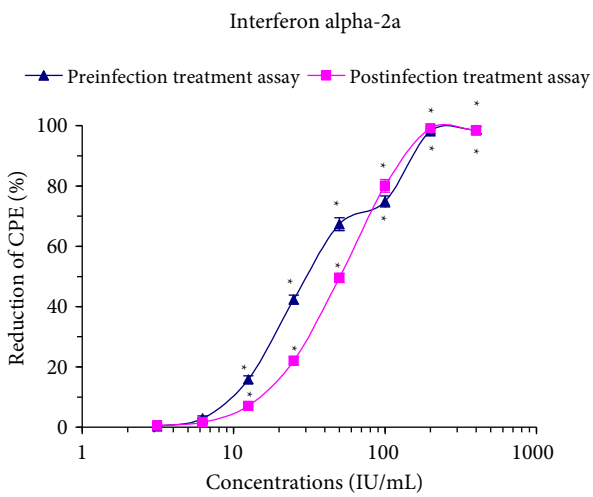

Figure 5. Reduction of poliovirus type 1-induced CPE after treatment with interferon α-2a (*: $P < 0.05$).

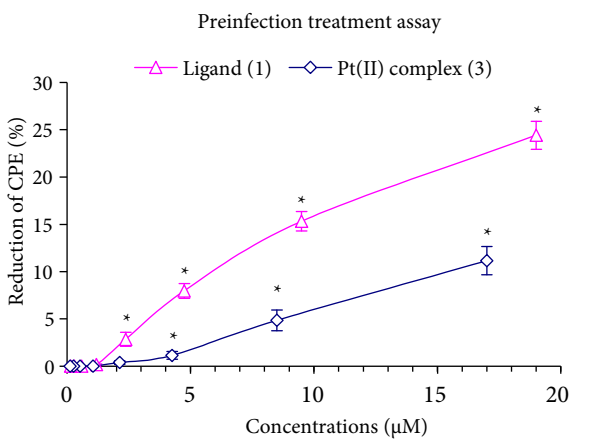

Figure 6. Reduction of poliovirus type 1-induced CPE in the preinfection treatment with compounds 1 and 3 (*: $P < 0.05$).

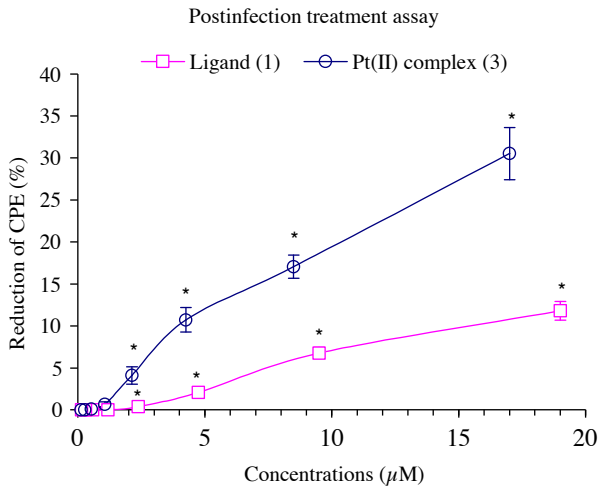

Figure 7. Reduction of poliovirus type 1-induced CPE in the postinfection treatment with compounds 1 and 3 (*: $P < 0.05$).

was detected at concentrations of 2.38 μM and higher, with the highest effect observed at 19 μM (11.8 ± 1.13% of CPE reduction). For the Pt(II) complex, a significant inhibition of the CPE was measured at concentrations of 2.13 μM and above, with the highest effect observed at 17 μM (30.52 ± 3.12% of CPE reduction). From these results it is clear that in the postinfection treatment the Pt(II) complex possessed a higher antiviral potential than the ligand.

4. Discussion

The development of biologically active agents that exploit differences between cancerous and normal cells, that possess a higher specificity for cancer cells, and that cause less damage to normal cells remains the ultimate objective in antineoplastic drug development (Adams, 2001). The cytotoxic activity of novel Pd(II) and Pt(II) complexes with the 2-(diphenylphosphino)benzaldehyde 1-adamantoylhydrazone ligand was previously analyzed in human larynx carcinoma cells (Hep-2) and healthy human lung fibroblast cells (MRC-5) (Đorđević et al., 2014). Previous results in Hep-2 cells showed that the Pt(II) complex and the ligand itself have cytotoxic activities within the range of oxaliplatin's activity. The MRC-5 cell line manifested high tolerance to the ligand and its metal complexes. These novel compounds are characterized with selective cytotoxic activity to cancerous Hep-2 cells in comparison to healthy MRC-5 cells.

The results obtained from the examination of cytotoxicity in A549 cells revealed that the ligand displays a dose-dependent cytotoxic activity that is 4 times weaker than that of cisplatin and oxaliplatin, but markedly higher than that of carboplatin. Comparison of the cytotoxicity results of A549 cells and the results obtained in our previous experiments in healthy lung MRC-5 cells (IC_{50} = 114.07 ± 5.11 μM) (Đorđević et al., 2014) revealed that the ligand does not display specificity to A549 cells. In contrast to the ligand, neither the Pt(II) nor the Pd(II) complex induced cell death in A549 cells at the tested range of concentrations. The cytotoxicity results indicate that the introduction of the metal into this ligand structure does not increase the agent's cytotoxicity in the A549 cell line.

Development of palladium(II)-containing compounds is based on the similarity of the coordination chemistry of Pt(II) and Pd(II) complexes. However, most of the palladium complexes are characterized by lower cytotoxicity in comparison to their platinum analogs, probably due to their faster hydrolysis, which results in highly reactive species that cannot reach their pharmacological targets (Abu-Surrah et al., 2008). In addition to the physicochemical properties of these compounds, the absence of cytotoxic activity of Pt(II) and Pd(II) complexes (compounds 2 and 3, respectively) in A549 cells could be related to the high resistance potential of these cells. This is mediated by mechanisms involved in resistance development, such as reduced cellular uptake, increased efflux, and increased DNA repair (Rabik and Dolan, 2007).

Morphological evaluation of A549 cells that were treated with the ligand revealed the presence of typical signs of early and late apoptosis, such as membrane blebbing, chromatin condensation, and formation of apoptotic bodies (Vejselova and Kutlu, 2015). Thus, the ligand can be considered an apoptosis-inducing agent. Complexes comprising Pt(II) and Pd(II) and the condensation product of 2-(diphenylphosphino)benzaldehyde (dpba) hydrazone and semioxamazide display strong cytotoxicity to cisplatin-resistant osteosarcoma U2-OS/Pt cells and induce apoptosis in HeLa cells without causing significant perturbations of the cell cycle (Maleševi ć et al., 2006). In view of the considerable similarities in the chemical structures of other dpba hydrazones and the ligand (compound 1), they probably exert their effects through the same mechanisms of action.

Assessment of the antiviral effect under different experimental conditions provides information as to the intracellular action of an agent at specific steps in the virus life cycle (Enan et al., 2012). Investigation of the antiviral activity of the Pd(II) complex revealed that this compound does not possess any antiviral activity against poliovirus type 1 at the level of viral adsorption and viral replication, as there was no observable reduction of CPE at any of the applied concentrations. Treatment of cells with the ligand and the Pt(II) complex prior to poliovirus type 1 infection resulted in some antiviral activity. The ligand expressed better antiviral activity than the Pt(II) complex. According to Šmidling et al. (2008) this probably resulted from the blockage of virus receptor binding sites on the

cell surface and decreased viral adsorption. Treatment of cells with the ligand and Pt(II) complex after infection with the poliovirus revealed a greater antiviral potential of the Pt(II) complex and its ability to reduce viral replication in comparison to the ligand. The decrease of viral activity induced by the Pt(II) complex was achieved by inhibition of viral replication.

Although we obtained significant inhibition of CPE with the ligand and Pt(II) complex, 50% of viral inhibition (the threshold antiviral activity recommended by the FDA) was not obtained. Therefore, relative to this recommendation, the ligand and Pt(II) complexes have weak antiviral activity.

Of the examined compounds, ligand 2-(diphenylphosphino) benzaldehyde 1-adamantoylhydrazone exhibits cytotoxic activity and induces morphological changes associated with apoptosis. Thus, it should be viewed as a potential anticancer agent. The ligand and the Pt(II) complex caused viral inhibition and should be considered for further investigation of antiviral activity.

Acknowledgments

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