BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20954

Article

A novel second-generation platinum derivative and evaluation of its anti-cancer potential

Habibe Yilmaz[1,2*], Şenay Hamarat Şanlier[1,2]

[1] Ege University Center for Drug Research & Development and Pharmaceutical Applications (ARGEFAR), Izmir, Turkey, [2] Ege University, Faculty of Science, Biochemistry Department, Izmir, Turkey

Cisplatin is the primary anti-cancer agent for the treatment of most solid tumors. However, platinum-based anti-cancer chemotherapy produces severe side effects due to its poor specificity. There are a broad interest and literature base for a novel mechanism of action on platinum derivatives. Additionally, combining cisplatin with histone deacetylase inhibitors (HDACi) such as 4-hydroxybenzoic acid derivatives showed promising results in treating solid tumors. Here we aimed to conjugate 4-hydroxybenzoic acid with platinum to obtain a novel platinum derivative that can overcome cisplatin resistance. Cis-4-hydroxyphenylplatinum(II)diamine compound was synthesized under mild conditions and characterized. Cytotoxicity assay was performed on SKOV3-Luc and A549-Luc cells. Hemocompatibility and serum protein binding analysis were performed. Treatment potential was evaluated in xenograft tumor models. Biodistribution was tested on tumor-bearing mice via Pt analysis in organs with ICP-MS, *ex vivo*. In this study, cis-4-hydroxyphenylplatinum (II) diamine was synthesized with a yield of 62%. The MTT assay on A549-Luc and SKOV3-Luc cell lines resulted in IC_{50} values of 17.82 and 7.81 µM, respectively. While tumor growth was continued in the control group, the tumor volume decreased in the treatment group. All results point to the conclusion that the new compound has the potential to treat solid tumors.

Keywords: Platinum-derivative. 4-hydroxybenzoic acid. Anti-cancer agent. Xenograft model. Lung cancer.

INTRODUCTION

Platinum (Pt)-based anti-cancer agents are primarily used in the treatment of various solid tumors. Cisplatin has been used in standard chemotherapy regimens as a single treatment or combined with other cytotoxic agents or radiotherapy. However, this suffers from limitations due to systemic toxicities that affect the effectiveness of the treatment. Over the past 40 years, advances in technology have allowed us to prepare thousands of platinum complexes to achieve better toxicological profiles and higher activity (Wang, Guo, 2013).

Platinum complexes with cis geometry have two amine non-leaving groups and two anionic labile groups, which are structural analogs of cisplatin (Ho, Au-Yeung Steve, To Kenneth, 2003). Once inside the cell, the labile groups are hydrolyzed and displaced by water molecules, gaining potential electrophilic property to react with any nucleophiles, arrest cell division and induce apoptosis. Besides, cisplatin shows an antineoplastic effect through pathways such as calcium signaling pathway, mitogenic protein kinase pathway, Akt pathway, etc., and increasing levels of cisplatin-dependent reactive oxygen species (Dasari, Tchounwou, 2014). At present, many research groups continue their work on discovering possible new mechanisms of action (Yuan *et al.*, 2016).

Inhibition of histone deacetylases (HDACs) gained interest in relapsing or refractory to classical chemotherapy. HDAC inhibitors induce hyperacetylation of histone and nonhistone proteins, leading to the inhibition of cell

***Correspondence:** H. Yilmaz. Center for Drug Research & Development and Pharmaceutical Applications (ARGEFAR). Ege University, 35100 Bornova-Izmir, Turkey. Phone: +90 533 084 6240. E-mail: yilmaz.habibe@hotmail.com. ORCID: 0000-0003-1106-0458. Pharmaceutical Biotechnology Department. Faculty of Pharmacy. Trakya University. Balkan Campus, 22030, Edirne/TURKEY

cycle progression and induction of apoptosis. The latest studies on 4-hydroxybenzoic acid as an HDAC inhibitor exhibited promising results against various cancer cell lines, including lung cancer (Wang *et al*., 2018; Seidel *et al*., 2014). More recently, Wang *et al*. 2012 showed that the depletion of HDAC6 in non-small cell lung cancer (NSCLC) cell lines H292 and A549 resulted in the sensitization of cells to cisplatin treatment. Their findings indicated that HDAC6 is significantly associated with cisplatin resistance in NSCLC and, HDAC6 is a potential novel therapeutic target for platinum-refractory NSCLC (Wang *et al*., 2012).

Many studies of the combination of cisplatin and HDACi have shown promising results. However, there are no previous reports in the literature on the anti-cancer effects of a platinum compound containing HDAC inhibitor, which is known to avoid cisplatin resistance. Therefore, our objective can be restated as to conjugate 4-hydroxybenzoic acid with platinum to obtain a novel platinum derivative that does not trigger cisplatin-resistance and evaluate its anti-cancer potential. The novel compound was characterized by TLC, UV-Vis spectophotometry, NMR, FT-IR, and HPLC, followed by cytotoxicity assessment in the cisplatin-resistant NSCLC cell line (A549-Luc) and ovarian cancer cell line (SKOV3-Luc). In addition, biocompatibility studies were carried out before proceeding with *in vivo* studies. After that, preliminary *in vivo* studies were performed using a xenograft tumor model generated with A549-Luc cells in nude mice. The obtained data demonstrated that the new compound has the potential to be used as an anti-cancer agent. The illustrated study can be seen in Figure 1.

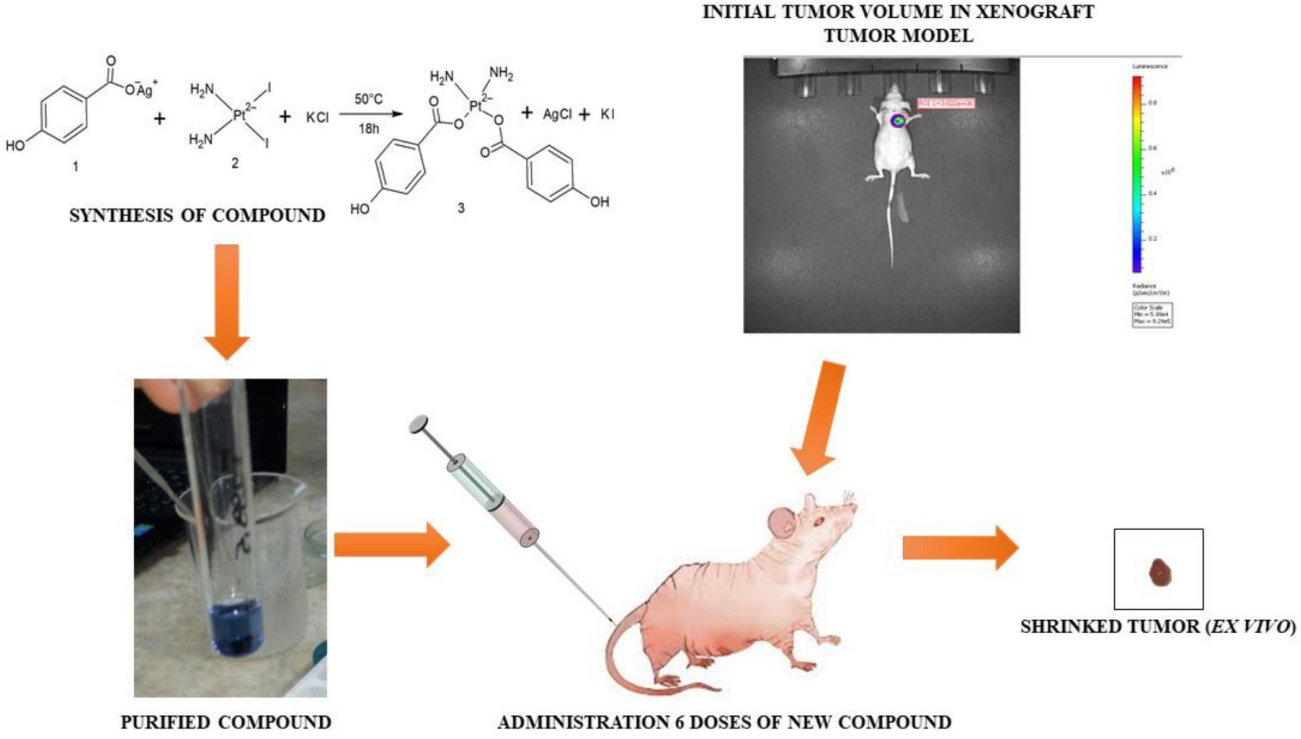


FIGURE 1 - Schematic illustration of the study. Synthesized new compound purified before in vitro and in vivo studies. Xenograft tumor model was created with A549-Luc cells in 5-6 weeks old Balb/c female nude mice, and tumor volumes were monitored during the study. After six doses of new compound administration, tumor volume decrease observed.

MATERIAL AND METHODS

Materials

4-hydroxybenzoic acid (HBA), N,N'-dimethylformamide(DMF), ethyl acetate, acetic acid, propanol, acetic acid, $Pt(NH_3)_2Cl_2$ from Sigma Aldrich, luciferin from BioVision, and Silica Gel 60 F254 aluminum TLC plates from MERCK, A549-Luc, and SKOV3-Luc cells were purchased from Perkin Elmer.

Synthesis and Characterization of Platinum Derivative

The synthesis was completed in 3 steps. First, 200 µmol HBA was dissolved in 20 mM $NaHCO_3$ solution. Next, 20 mM $AgNO_3$ solution was added and left to react for two h at RT. Second, in a different reaction vessel, 60 mg $Pt(NH_3)_2Cl_2$ was suspended in 20 mM KI solution and left to react for one h at 30°C. Third, HBA and $Pt(NH_3)_2Cl_2$ solutions were mixed and left to react for 18 h at 50°C. Upon completion of the reaction, AgCl precipitates were removed by centrifugation at 9000 rpm and supernatant dried at 50°C (Liu *et al*., 2012). The dried product was dissolved in DMF and purified with Silica Gel 60 resin. The mobile phase was 2-propanol: water: glacial acetic acid (13:5:3). The flow rate was adjusted to 1.5 mL/min, and fractions (1 mL) were collected (Chen *et al*., 2002; Pasini *et al*., 1993).

Quality control was performed with TLC, NMR, HPLC, UV-Vis spectrophotometry, and FT-IR. In TLC analysis, compounds were applied on Silica Gel 60 F254 aluminum TLC plates and placed in ACN: Ethyl acetate: chloroform (13:5:3) containing 0.1% glacial acetic acid solvent system. Lichrospher RP18-15 (Dimensions: 25 cm x 4,6 mm (internal diameter) id.) column and 15% acetonitrile (ACN, A), 0.5% o-phosphoric acid-water (85%, B) mobile phases were used under isocratic conditions for HPLC analysis. Ten µL reaction mixture was injected at a flow rate of 1 mL/min at 25°C. Detection was carried out at 254 nm (Dhanani, Shah, Kumar, 2015).

To calculate the reaction yield, the supernatant of the reaction medium was spectrophotometrically analyzed with the method previously described elsewhere to measure $Pt(NH_3)_2Cl_2$ concentration (Anilanmert *et al*., 2001).

Biocompatibility Studies of cis-4-hydroxyphenylplatinum(II)diamine

Determination of the amount of binding to serum proteins and hemolysis

A combination of Cole *et al*. and Semete *et al*.'s methods have been used to determine protein binding rates (Cole *et al*., 2011; Semete *et al*., 2012).

The mixture of cis-4-hydroxy phenyl platinum (II) diamine and FBS was prepared at a total volume of 300 µL in the ratio of 10:90, 20:80, 40:60, 60:40 and, 90:10 (v/v). It was incubated for 2 hours at 37°C and centrifuged at 13000 rpm for 15 min. The protein amount was measured by Bradford protein assay (Bradford, 1976).

To determine the amount of hemolysis, the combination of Mayer *et al.* and Yallapu *et al*.'s methods have been used (Mayer *et al*., 2009; Yallapu *et al*., 2015).

Blood was collected from Balb/c mice into EDTA tubes and pooled for hemolysis analysis (Ethical approval number: 2017-016). Collected blood was centrifuged at 1000 rpm for 10 minutes, and the plasma and buffy coats were removed from the erythrocytes. The erythrocytes were washed twice with 1X PBS and adjusted to a 2% hematocrit level. The experimental setup was prepared in 250, 100, 50, 25, and 10-fold dilutions using the compound at a 25 µg/mL concentration and erythrocyte suspension. Samples were incubated at 37°C for 2 hours. Erythrocytes were mixed with 1X PBS and 1% Triton X-100 to obtain negative and positive controls. At the end of the period, mixtures were centrifuged at 2000 rpm for 5 minutes. Hemoglobin content was measured spectrophotometrically at 540 nm. The hemolysis rate was calculated using the equation below:

$$\text{Hemolysis}(\%) = \frac{\text{Absorbance (sample)} - \text{Absorbance(negative control)}}{\text{Absorbance (positive control)}} \times 100$$

In Vitro Cell Culture Studies

The maintenance of cell lines and cultures

Cells were incubated in McCoy's or DMEM Ham's F12 (10% FBS, 1% L-glutamine, 1% gentamicin, and one mM HEPES) media in a CO_2 incubator at 37°C. Cells were

subcultured twice a week to obtain sufficient cell stocks and, cells that were in the logarithmic phase were used.

Cytotoxicity Assay

For the MTT assay, 10.000 cells of A549-Luc and SKOV3-Luc cells per well were inoculated in 96-well plates. Cis-4-hydroxyphenylplatinum(II)diamine at concentrations of 3.125, 6.25, 12.5, 25, 50, 100 and 200 µM were added. PBS, medium, and 200 µM cisplatin were used as controls. Cells were incubated for 24, 48, and 72 hours. At the end of the period, MTT was added, and cells were incubated for additional 4 hours. The resulting crystals were dissolved in DMSO and measured at 540 nm (Polarstar Omega). IC_{50} values and standard deviations were calculated via GraphPad Prism Software 8.0.

Evaluation of Biodistribution and Treatment Potential

Evaluation of Treatment Potential In vivo

In vivo and *ex vivo* studies were carried out in accordance with the Ege University Experimental Animal Ethics Committee approval (Approval Number is 2017-016).

To determine the therapeutic potential of cis-4-hydroxyphenylplatinum(II)diamine, a xenograft tumor model was established with A549-Luc cells in 5-6 weeks old Balb/c female nude mice. $6x10^6$ cells were injected between the two scapulae or coxae regions of the animals. Tumor volumes were measured with IVIS Spect System (745ex/820em) (Perkin Elmer IVIS Spectrum Imaging System) by subcutaneous injection of 100 µL luciferin (12 mg/mL in pH 7.4 PBS). Tumor volumes were calculated as follows:

$$\text{Tumor volume } (mm^3) = \frac{((Tumor\ width)x\ (Tumor\ width)\ x\ (Tumor\ lenght)]}{2}$$

Animals with tumor size above 200 mm^3 were grouped. The new compound prepared in the sterile saline (0.9% NaCl) solution was injected through the tail vein at a 20 mg/kg dose (n=4). The mice in the control group (n=3) were received only PBS.

Evaluation of Biodistribution Ex Vivo

To determine the biodistribution, 20 mg/kg new compound was administered to the control group through the tail vein. Three hours post-administration, mice were sacrificed using ketamine/xylazine overdose.

Within the scope of *ex vivo* studies, lungs, liver, kidney, spleen, and tumors were collected and degraded chemically. The washed, dried, and weighed organs were frozen in 1 mL of pH 7.4 phosphate buffer, then thawed and homogenized with a sonic homogenizer. The homogenized samples were taken into Folin tubes, and 9 mL of HNO_3:$HClO_4$ (5:1) was added (Alhareth *et al.*, 2012; Esteban-Fernandez *et al.*, 2008). The mixture was degraded for 30 min and ICP-MS analysis was performed for Pt content determination.

All experiments were performed in triplicate for each sample except *in vivo* studies. Descriptive statistics were calculated for the data obtained (mean ± standard deviation). The comparison of the means between the groups was made by ANOVA ($p < 0.05$). Statistical analysis of the *in vivo* study results was performed using a nested t-test ($p < 0.05$).

RESULTS AND DISCUSSION

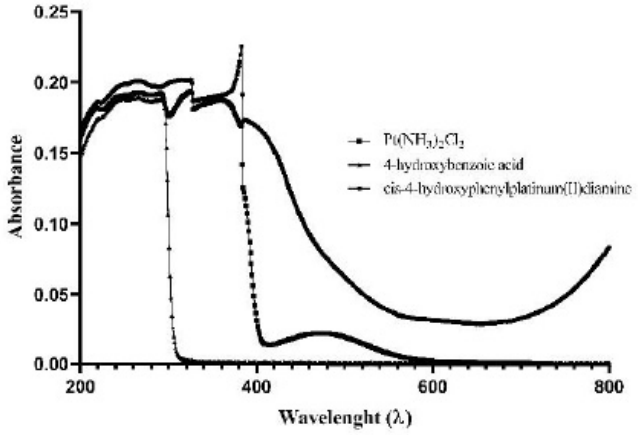
Synthesis and Characterization of cis-4-hydroxyphenylplatinum(II)diamine

The outcome of various experimentation leads to the conclusion that a novel second-generation platinum derivative was successfully synthesized. The synthesis was completed in 3 steps and purified by the silica gel 60 columns. Purified cis-4-hydroxyphenylplatinum(II)diamine was characterized by TLC, HPLC, NMR, FT-IR, and UV/VIS spectrophotometer. TLC results and wavelength scan of starting materials and obtained compound can be seen in Figure 2(A) and Figure 2(B), respectively.

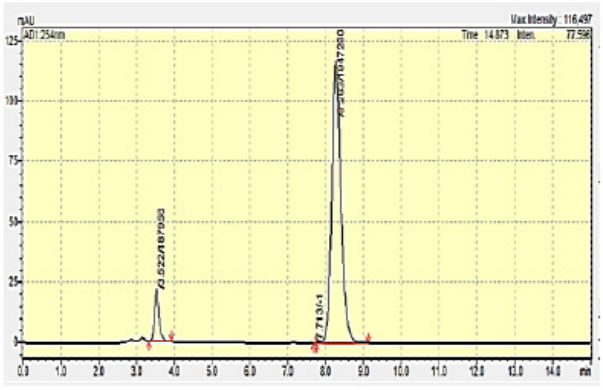
A

Sample	R_f1	R_f2
HBA	0.53	-
HBA-Ag	0.51	-
cis-4-hydroxyphenylplatinum (II)diamine	0.49	0.05

B



C



D

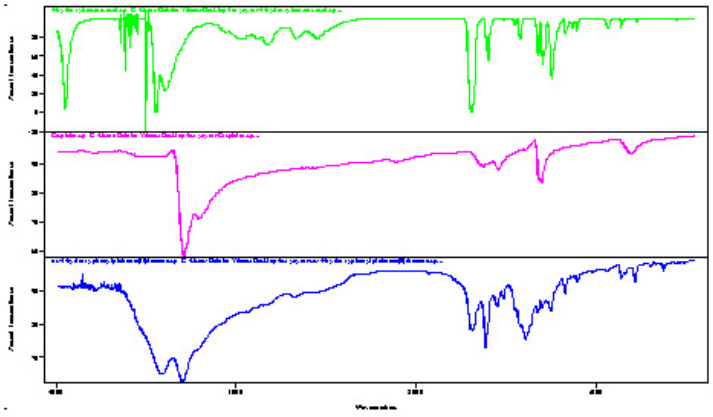


FIGURE 2 - A) TLC analysis results of cis-4-hydroxyphenylplatinum(II)diamine, HBA and HBA mixed with silver nitrate. The increased number of apolar groups in the new compound compared to HBA caused the Rf value to be lower than the starting compounds with 0.49. **B)**UV/Vıs spectrum of cis-4-hydroxyphenylplatinum (II) diamine and initial compounds HBA and $Pt(NH_3)_2Cl_2$. HBA displayed absorption in UV range while $Pt(NH_3)_2Cl_2$ displayed both in UV and Vis range. Thus, the new compound displayed absorption both in UV and Vis range with an absorption maximum. **C)** HPLC result of reaction medium upper phase revealed that there are two peaks at 3.5 minutes and 8.2 minutes belong to cis-4-hydroxyphenylplatinum(II) diamine and HBA, respectively, **D)** FTIR analysis result of HBA [top], cis-4-hydroxyphenylplatinum(II)diamine [bottom] and $Pt(NH_3)_2Cl_2$ [middle]. The primary amine group and Pt signal in $Pt(NH_3)_2Cl_2$, and the aromatic ring in HBA were also observed in cis-4-hydroxyphenylplatinum(II)diamine. Thus, the overall data supported the success of the synthesis.

HPLC analysis was performed based on the previously published study (Dhanani, Shah, Kumar, 2015). As shown from Figure 2(C), there are two peaks in chromatogram at 3.5 minutes and 8.2 minutes, belonging to cis-4-hydroxyphenylplatinum(II)diamine 4-hydroxybenzoic acid, respectively. To calculate the reaction yield, unreacted $Pt(NH_3)_2Cl_2$ was measured spectrophotometrically, and the overall reaction yield was calculated as 62.7% (Anilanmert *et al.*, 2001).

FT-IR analysis was performed to confirm the structures of compounds. Both HBA, $Pt(NH_3)_2Cl_2$, and cis-4-hydroxyphenylplatinum(II)diamine were analyzed with FT-IR, and the results were compared. The results can be seen in Figure 2(D). The peaks of primary

amine groups for $Pt(NH_3)_2Cl_2$ were found at 3472.78 and 3201.91 cm^{-1}. However, there was no absorption in the primary amine of the HBA compound as expected. The peaks belong to the C=O, and C-O bonds of HBA were found at 1725-1705 cm^{-1}. The aromatic ring peaks which belong to the C=C bond were recorded at 1593.99 cm^{-1}, 1509.29 $cm^{-1,}$ and 1419.57 cm^{-1}. FT-IR spectrum of cis-4-hydroxyphenylplatinum(II)diamine revealed that the peaks of C-H and the primary amine group in the aromatic ring overlapped at 3400 3286.70 cm^{-1}. The carbonyl group peak was observed at 1682.2 $cm^{-1,}$ and the signals belong to the C-O bond are observed at 1241.04 cm^{-1} and 1167.77 cm^{-1}. The carbonyl group peak was shifted after the reaction and detected at 1676.54 cm^{-1}. The shifted C=C signals of the aromatic rings in cis-4-hydroxyphenylplatinum(II)diamine were found at 1608.95 cm-1, 1542.12 cm^{-1}, and 1510.33 cm^{-1}. The peak recorded at 795.64 cm^{-1} was thought to belong Pt-Cl bond in $Pt(NH_3)_2Cl$. On the other hand, it was shifted to 778.83 cm^{-1}, suggesting that a new chemical entity was added to the structure. Based on the FT-IR analysis, the synthesis was performed successfully.

To further confirm cis-4-hydroxyphenylplatinum(II) diamine structure, C^{13} and H^1 NMR analyses were performed. NMR spectra of a new compound and other compounds can be seen in Figure 3.

As seen from the H^1 NMR spectrum of cis-4-hydroxyphenylplatinum(II)diamine in Figure 3(A), the signal at 6.7 ppm gave pentate, which means there is 4 H in the aryl structure. Also, there is a triplet signal at 7.8 ppm, which means there are 2 two equal protons in the structure, indicating the hydroxy groups bound to aryl. At four ppm, characteristic signal as singlet belongs to amine in platinum structure can also be seen. The signals at 2.5 ppm and 2.8 ppm belong to DMSO-d_6 and residual DMF, respectively. In cis-4-hydroxyphenylplatinum(II)diamine, there are four different types of carbon that are significant in terms of C^{13} NMR, and different signals are expected for these carbons (Figure 3(B)). The carbons found in the aromatic ring gave signals at about 115 ppm and 131 ppm. Two different signals are seen because the two carbons are not equivalent. Carbons in aromatic rings give different signals because one is neighbor to the carbonyl group and the other one is to the hydroxyl group. Carbonyl group carbon gave a signal at 169 ppm, and the carbon which is neighbor to the hydroxyl group at 161 ppm. The signal at 163 ppm belongs to residual DMF, and the signal at 40 ppm belongs to DMSO-$d_{6.}$

As seen from Figure 3(C), the proton signal belonging to the carboxy group of HBA at 12.3 ppm is lost after reaction, confirming that the compound is synthesized. On the other hand, the signals of the amine group, which belongs to $Pt(NH_3)_2Cl_2$ (in Figure 3(D)) can also be seen in cis-4-hydroxyphenylplatinum(II)diamine, which is strong evidence that the reaction was successful.

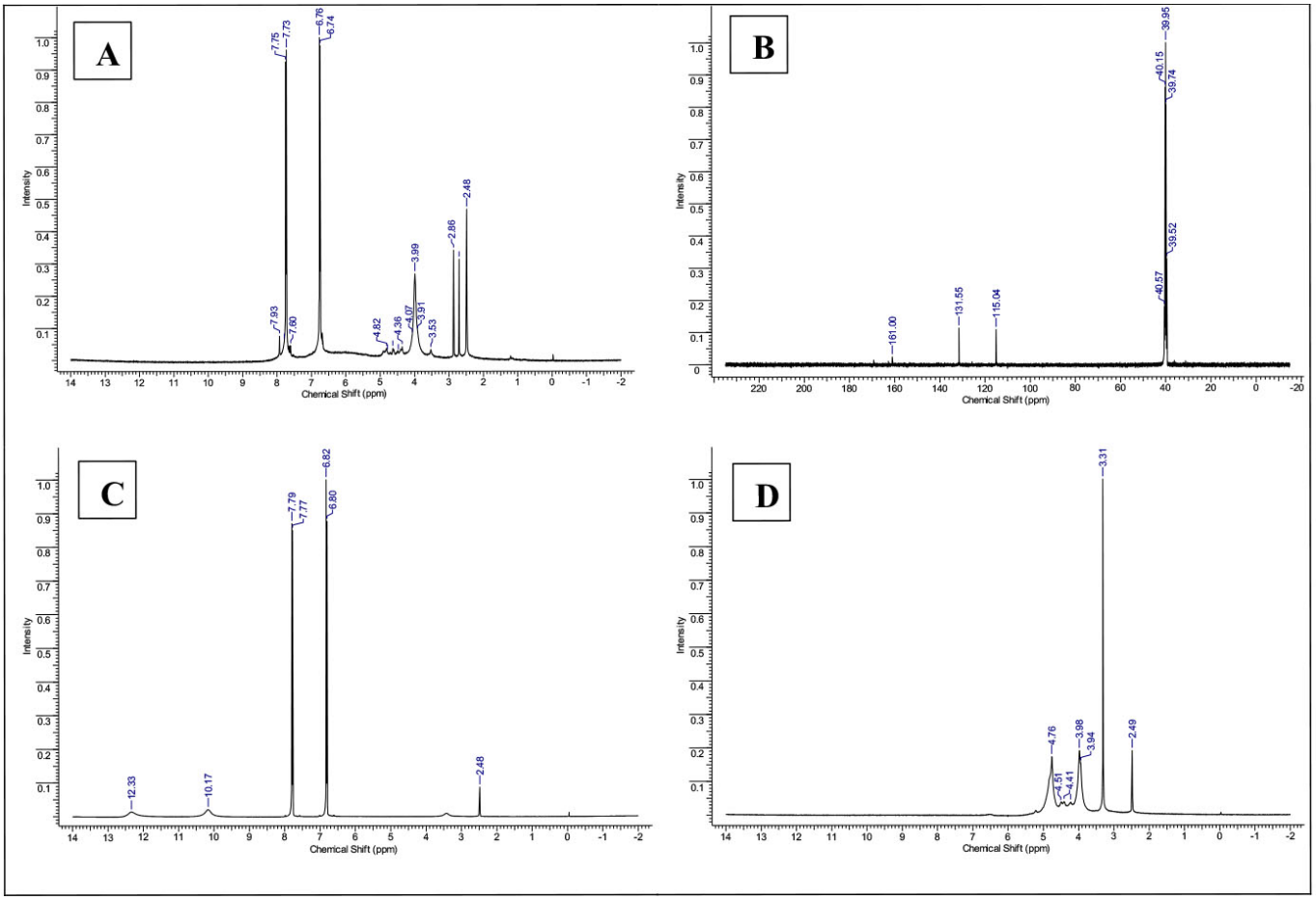


FIGURE 3 - A) H^1 NMR spectra of cis-4-hydroxyphenylplatinum (II) diamine, **B)** C^{13} NMR spectra of cis-4-hydroxyphenylplatinum(II)diamine **C)** H^1 NMR spectra of HBA and **D)** H^1 NMR spectra of $Pt(NH_3)_2Cl_2$. Protons in primary amine and aromatic ring and carbonyl protons were detected in $Pt(NH_3)_2Cl_2$, cis-4-hydroxyphenylplatinum(II)diamine, and HBA, respectively, in H1 NMR analysis. In the C^{13} NMR analysis of cis-4-hydroxyphenylplatinum(II)diamine aromatic ring carbons and carbonyl group carbon were detected, which proved the overall structure contains an aromatic ring carbonyl group and a primary amine.

Biocompatibility Studies of cis-4-hydroxyphenylplatinum (II) diamine

Determination of the amount of binding to serum proteins and hemolysis

Drugs that are administered from the iv route are generally eliminated in 2 hours. Therefore, the incubation time was chosen as 2 hours. In addition, the incubation was carried out at 37°C to simulate the body temperature. All samples were tested in triplicate. The obtained results can be seen in Figure 4.

According to free drug theory (FDT), the free concentration of a drug can increase due to the variances in serum protein levels, resulting in the requirement of drug dosage adjustment (Bohnert, Gan, 2013). As seen from Figure 4(B), the rate of binding of the new compound to serum proteins was low and not highly affected by the change in serum protein content. Based on the previous findings, the percentage of cisplatin binding to albumin and rat serum proteins was approximately 92%, since cisplatin can easily coordinate with proteins due to very exposed platinum core (Cole, Wolf, 1980). On the other hand, it is unsurprising to find that there is a considerably lower protein binding rate of the new compound since the platinum core is more concealed.

Hemolysis measurement is a standard method used to test membrane permeabilization activities of drugs.

Hemolysis is usually caused by RBC swelling due to the formation of pores or ducts in the plasma membrane (Arias *et al*., 2010).

Pore formation is the result of the interaction of drugs with membrane lipids and the formation of ion-conducting pores, resulting in aggregated structures on the membrane surface. As a result, RBCs burst due to fluid entry into the cell, and hemoglobin leakage occurs (Aranda, Teruel, Ortiz, 2005). Based on the obtained data, which can be seen in Figure 4(A), the increasing amount of the new compound was not change the ratio of hemolysis and was found as 3.5%.

In 2014, Kutwin *et al*. investigated the level of hemolysis and structural deterioration of cisplatin using red blood cells obtained from chicken. They found the ratio of cisplatin hemolysis as 14%. In cis-4-hydroxyphenylplatinum(II)diamine, chloride was replaced with 4-hydroxybenzoic acid and covalently conjugated to platinum. Therefore, no hydrolyzable groups are resulting in electrophiles that can interact with the membrane and create hemolysis in cis-4-hydroxyphenylplatinum(II)diamine. As a consequence, a decrease in hemolysis compared to cisplatin was observed as expected.

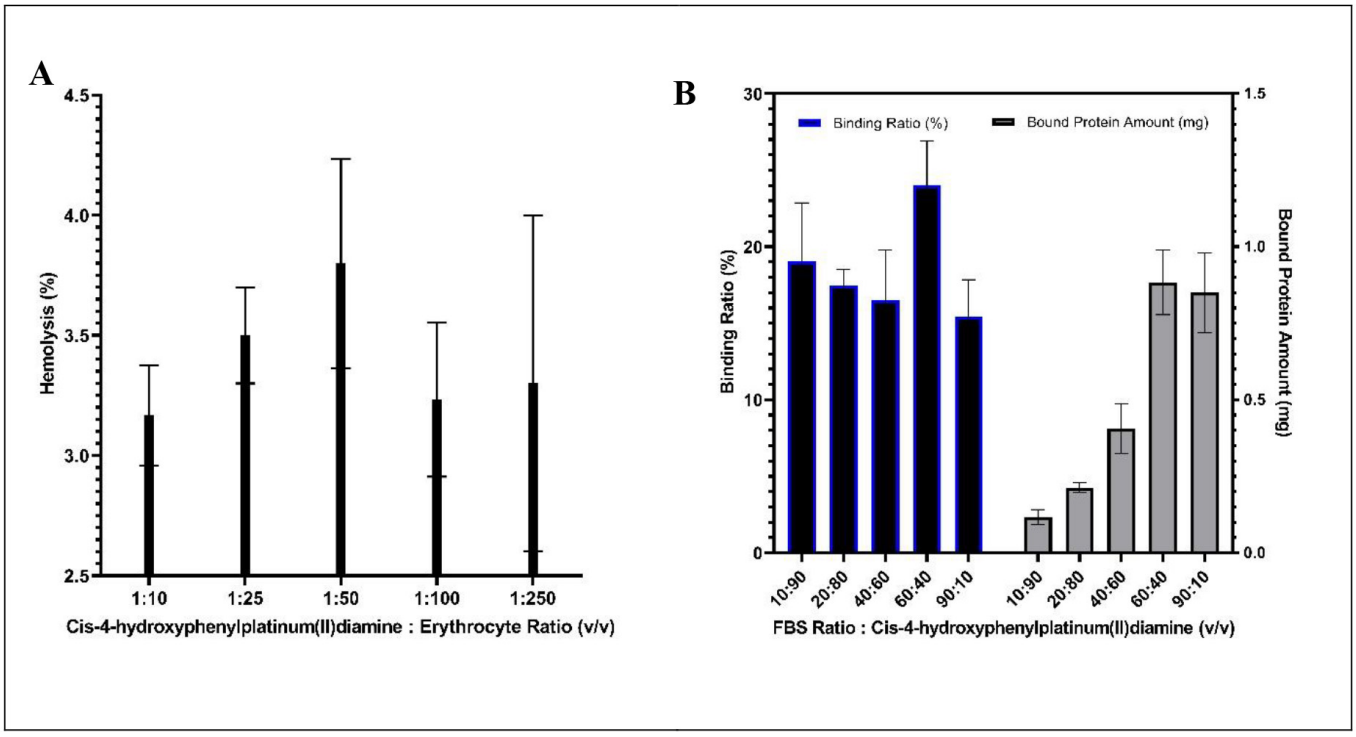


FIGURE 4 - A) % Hemolysis rates obtained from new compound treated with erythrocyte cells in varying proportions ($p<0.05$). cis-4-hydroxyphenylplatinum (II) diamine compound caused slight hemolysis with a 3.5%±1 ratio. **B)** Serum protein binding ratio of a new compound ($p<0.05$). The protein binding ratio was almost constant with the varying FBS: cis-4-hydroxyphenylplatinum (II) diamine ratio. Thus, the new compound's binding rate to serum proteins is low and is not highly affected by the change in serum protein content.

Cytotoxicity Assay

Cisplatin and other platinum derivatives are generally used to treat various solid tumors, including lung and ovarian cancer. Since the compound is a platinum derivative, it is expected to be effective on solid tumors. Besides, chlorides in the cisplatin structure were replaced by 4-hydroxybenzoic acid, which prevents cisplatin resistance by inhibiting HDAC. Therefore, its cytotoxicity was evaluated on SKOV3-Luc and A549-Luc cell lines. A549-

Luc cells are known to be resistant to platinum derivatives. Both cells were treated with varying concentrations of the new compound for 24, 48, and 72 hours before the MTT test. Obtained results can be seen in Table I.

Based on the obtained data, the new compound's IC_{50} value for SKOV3-Luc cells was 7.81 μM and 17.82 μM for A549-Luc cells. As can be seen from Table I, the effect of cis-4-hydroxyphenylplatinum(II)diamine was more time-dependent for A549-Luc cells. This effect may be due to the platinum resistance of the cells.

TABLE I - IC_{50} values obtained at 24th, 48th and 72th hours after new compound applied MTT test performed in A549-Luc and SKOV3-Luc cell lines

Time (h)	IC_{50} (**A549**-luc, μM)	IC_{50} (**SKOV3**-luc, μM)
24	106.40 ± 1.05	28.98 ± 1.08
48	20.04 ± 1.09	7.90 ± 1.04
72	17.82 ± 1.09	7.81 ± 1.04

*Descriptive statistics were calculated for the data obtained (mean ± standard deviation). Each group studied in 10 replicates.

Evaluation of Biodistribution and Treatment Potential

The administration dose was chosen based on literature data of cisplatin and carboplatin doses. Literature data showed that *in vivo* administration doses for both cisplatin and carboplatin were quite variable. Therefore, Caffrey and Frenkel were used a 15 mg/kg initial treatment dose of carboplatin. Following the first dose, they continued treatment with either 50 mg/kg carboplatin or 7.2 mg/kg cisplatin (Caffrey, Frenkel, 2013). To determine the cisplatin toxicity, rats were administered 20 mg/kg cisplatin (Price *et al.*, 2006). In another study, cisplatin was administered at a dose of 10 mg/kg dose in iv. route and carboplatin were administered at a dose of 120 mg/kg to female Balb/c mice (McKeage *et al.*, 1993).

Since the literature data is quite variable, we have calculated the LD_{50} dose of the new compound based on Wong *et al.*'s 2017 study and found it as 249.1 mg/kg. Since the new compound can be classified as the second generation platinum derivative, it is concluded that the application dose should be higher than the first generation platinum derivative cisplatin dose. Therefore, it was decided to use a 20 mg/kg application dose which is almost 12.5 fold below the estimated LD_{50} dose. The initial IVIS-Spectrum images of mice and both initial and final tumor volumes can be seen in Figure 5.

The mice have received six doses of new compound twice weekly. Following the completion of the study, tumor volumes were measured with a manual caliper since fluorescent signals could not be received from tumors. This is because platinum derivatives cause DNA damage and form reactive oxygen species. Therefore, it is possible to lose the fluorescence signal. Also, it is a known fact that genetically modified cells can lose their luciferase activity under stress conditions (Czupryna, Tsourkas, 2011). The tumor volumes of all the treated mice were decreased by nearly 97%. Mice treated with cis-4-hydroxyphenylplatinum(II)diamine were gained weight between 0.4% to 16.9%.

On the other hand, tumor volumes of the control group were nearly unchanged or increased. The weight loss of the control group was between 8.3% to 9.6%. Tumor volume change data were analyzed with nested t-test, and obtained p-value was found as 0.0064, which indicated that tumor volume decrease was significant.

Biodistribution was performed in the control group following the completion of the treatment study. Single-dose of the compound was injected through the tail vein. After 3 hours, mice were sacrificed, and organs were collected for ICP-MS analysis. The analysis results can be seen in Figure 5(C).

Weight alteration of the mice was monitored twice weekly for both groups. Obtained data can be seen from Table II.

Based on the biodistribution data, cis-4-hydroxyphenylplatinum(II)diamine was accumulated intensively in the liver after 3 hours, indicating that it was metabolized in the liver. It was then mostly accumulated in kidneys, increasing the possibility of nephrotoxicity of cis-4-hydroxyphenylplatinum(II)diamine similar to other platinum derivatives. It was estimated that the low amount

of platinum in the spleen is due to the inability of the spleen to take an active role in the metabolism of small molecule drugs. The platinum compound was also detected in the tumor higher than that detected in the spleen.

The biodistribution showed that the new compound was metabolized in the liver and cleared via kidneys. To better understand the treatment potential, it was tested on Balb/c nude mice xenograft tumor models. Following the administration of six doses to mice, a significant tumor volume decrease was observed. Based on those preliminary findings, it can be suggested that the novel compound has the potential as an anti-cancer agent.

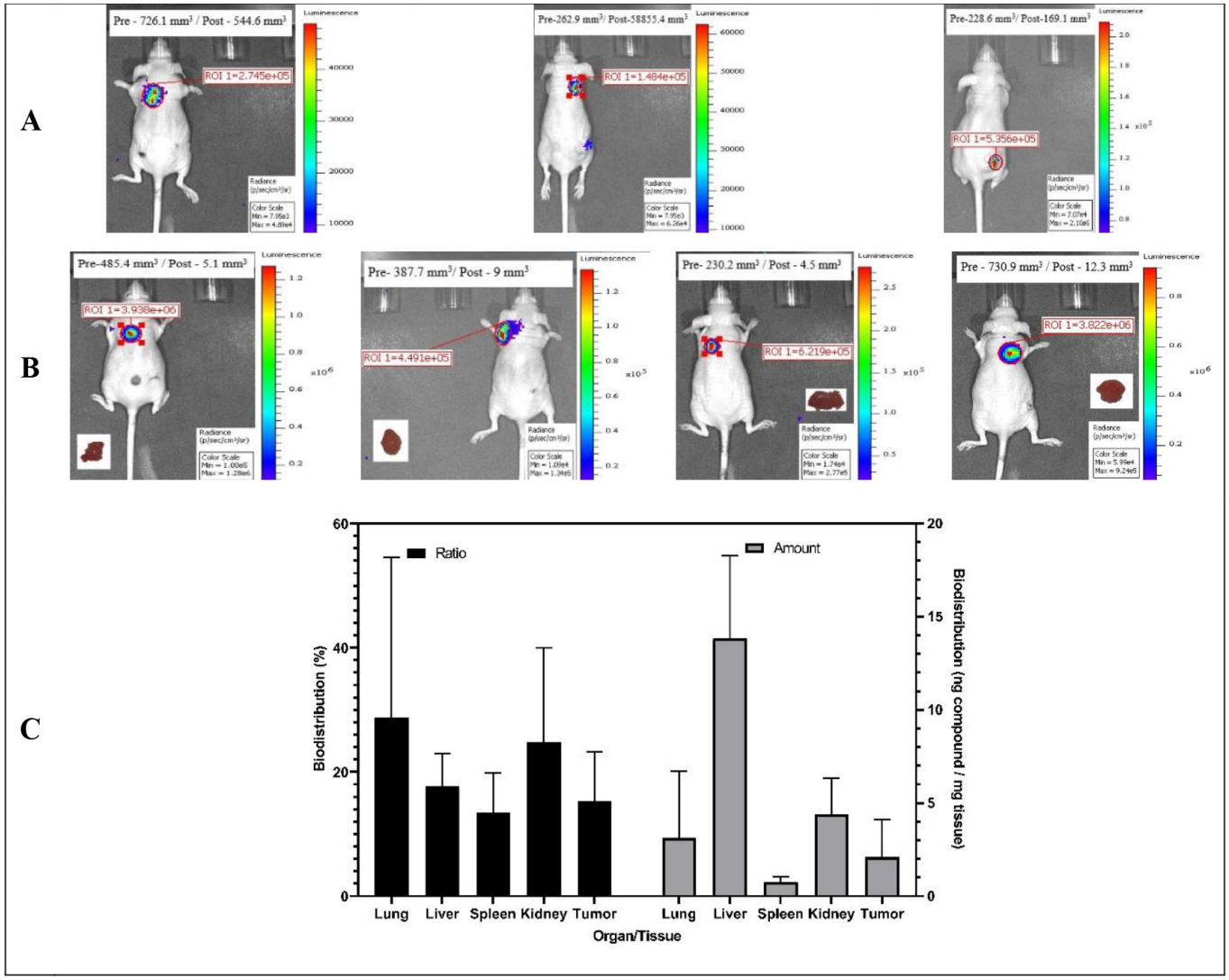


FIGURE 5 - A) Initial IVIS-Spect images and tumor volumes of the control group. (Pre: initial tumor volume and Post: tumor volume on the final day of the experiment). The tumor volumes of the control group remained unchanged or increased during the study. **B)** Initial IVIS-Spect images, initial and final tumor volumes, and tumor images of new compound-treated mice. After 6 six doses of new compound administration, the tumor volume decreased significantly. **C)** Biodistribution study results obtained via ICP-MS analysis ($p<0.05$). The new compound was distributed to the whole body well. A high amount in the liver and kidney was observed. The new compound was also detected in the tumor higher than that detected in the spleen.

TABLE II - Tumor volume and weight alteration of treatment and control groups

Group	Initial Tumor Volume (mm^3)	Final Tumor Volume (mm^3)	Initial Weight (g)	Weight after tumor model (g)	Percent Change (%)	After Treatment (g)	Percent Change (%)
Treatment Group	730.9	12.3	28	22	-21.4	24	+9.1
	485.4	5.1	24	20.9	-12.9	21	+0.5
	387.7	9.0	24.1	22.6	-6.2	22.7	+0.4
	230.2	4.5	23.9	23	-3.8	23.1	+0.4
Control Group	228.6	169.1	26	26	0		
	262.9	58855.4	27	24.4	-9.6		
	726.1	544.6	26.5	24.3	-8.3		

*Descriptive statistics were calculated for the data obtained (mean ± standard deviation). n=4 for treatment group and 3 for control group.

ACKNOWLEDGMENT

This publication was produced from Habibe YILMAZ's doctoral dissertation and financially supported by Ege University Scientific Research Projects Office with the project number 16 FEN 007.

REFERENCES

Alhareth K, Vauthier C, Gueutin C, Ponchel G, Moussa F. HPLC quantification of doxorubicin in plasma and tissues of rats treated with doxorubicin loaded poly(alkylcyanoacrylate) nanoparticles. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;887-888**:**128-132.

Anilanmert B, Yalcin G, Arioz F, Dolen E. The spectrophotometric determination of cisplatin in urine, using o-phenylenediamine as derivatizing agent. Anal Lett. 2001;34(1)**:**113-123.

Aranda FJ, Teruel JA, Ortiz A. Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. Biochim Biophys Acta Biomembr. 2005;1713(1):51-56.

Arias M, Quijano JC, Haridas V, Gutterman JU, Lemeshko VV. Red blood cell permeabilization by hypotonic treatments, saponin, and anti-cancer avicins. Biochim Biophys Acta. 2010;1798(6):1189-1196.

Bohnert T, Gan LS. Plasma protein binding: From discovery to development. J Pharm Sci. 2013;102(9)**:**2953-2994.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.

Caffrey PB, Frenkel GD. Prevention of carboplatin-induced resistance in human ovarian tumor xenografts by selenite. Anticancer Res. 2013;33(10):4249-54.

Chen Y, Janczuk A, Chen X, Wang J, Ksebati M, Wang PG. Expeditious syntheses of two carbohydrate-linked cisplatin analogs. Carbohydr Res. 2002;337(11)**:**1043-6.

Cole AJ, David AE, Wang J, Galbán CJ, Hill HL, Yang VC. Polyethylene glycol modified, cross-linked starch-coated iron oxide nanoparticles for enhanced magnetic tumor targeting. Biomaterials. 2011;32(8):2183-2193.

Cole WC, Wolf W. Preparation and metabolism of a cisplatin/serum protein complex. Chem Biol Interact. 1980;30(2):223-235.

Czupryna J, Tsourkas A. Firefly Luciferase and Rluc8 Exhibit Differential Sensitivity to Oxidative Stress in Apoptotic Cells. PLoS One. 2011;6(5):e20073.

Dasari S, Tchounwou PB. Cisplatin in cancer therapy: Molecular mechanisms of action. Eur J Pharmacol. 2014;740:364-378.

Dhanani T, Shah S, Kumar S. A validated high performance liquid chromatography method for determination of three bioactive compounds p-hydroxy benzoic acid, negundoside and agnuside in Vitex species. Maced J Chem Chem Eng. 2015;34(2):321-331.

Esteban-Fernandez D, Verdaguer JM, Ramirez-Camacho R, Palacios MA, Gomez-Gomez MM. Accumulation, fractionation, and analysis of platinum in toxicologically affected tissues after cisplatin, oxaliplatin, and carboplatin administration. J Anal Toxicol. 2008;32(2):140-6.

Ho YP, Au-Yeung CFS, To Kenneth KW. Platinum-based anti-cancer agents: Innovative design strategies and biological perspectives. Med Res Rev. 2003;23(5):633-655.

Kutwin M, Sawosz E, Jaworski S, Kurantowicz N, Strojny B, Chwalibog A. Structural damage of chicken red blood cells exposed to platinum nanoparticles and cisplatin. Nanoscale Res Lett. 2014;9(1):257-257.

Liu W, Chen X, Ye Q, Hou S. 3-Hydroxycarboplatin, a simple carboplatin derivative endowed with an improved toxicological profile. Platinum Met Rev. 2012;56(4);248-256.

Mayer A, Vadon M, Rinner B, Novak A, Wintersteiger R, Fröhlich E. The role of nanoparticle size in hemocompatibility. Toxicology. 2009;258(2-3):139-147.

McKeage MJ, Morgan SE, Boxall FE, Murrer BA, Hard GC, Harrap KR. Lack of nephrotoxicity of oral ammine/amine platinum (IV) dicarboxylate complexes in rodents. Br J Cancer. 1993;67(5):996-1000.

Pasini A, Caldiroia C, Spinelli S, Valsecchi M. Comments on Different Synthetic Methods for the Preparation of Diammine and bis(Amine) Organodicarboxylatoplatinum(II) Complexes. Synth React Inorg Met -Org Chem. 1993;23(6):1021-1060.

Price PM, Yu F, Kaldis P, Aleem E, Nowak G, Safirstein RL, et al. Dependence of cisplatin-induced cell death in vitro and in vivo on cyclin-dependent kinase 2. J Am Soc Nephrol. 2006;17(9):2434-42.

Seidel C, Schnekenburger M, Dicato M, Diederich M. Antiproliferative and proapoptotic activities of 4-hydroxybenzoic acid-based inhibitors of histone deacetylases. Cancer Lett. 2014;343(1):134-46.

Semete B, Booysen L, Kalombo L, Ramalapa B, Hayeshi R, Swai HS. Effects of protein binding on the biodistribution of PEGylated PLGA nanoparticles post oral administration. Int J Pharm. 2012;424(1-2):115-120.

Wang L, Xiang S, Williams KA, Dong H, Bai W, Nicosia SV, et al. Depletion of HDAC6 enhances cisplatin-induced DNA damage and apoptosis in non-small cell lung cancer cells. PLoS One. 2012;7(9):e44265.

Wang X, Guo Z. Targeting and delivery of platinum-based anti-cancer drugs. Chem Soc Rev. 2013;42(1):202-224.

Wang XN, Wang KY, Zhang XS, Yang C, Li XY. 4-Hydroxybenzoic acid (4-HBA) enhances the sensitivity of human breast cancer cells to adriamycin as a specific HDAC6 inhibitor by promoting HIPK2/p53 pathway. Biochem Biophys Res Commun. 2018;504(4):812-819.

Wong TS, Hashim Z, Zulkifli RM, Ismail HF, Zainol SN, Md Rajib NS, et al. LD_{50} Estimations for Diabecine™ Polyherbal Extracts Based on In Vitro Diabetic Models of 3T3-L1, WRL-68 and 1.1B4 Cell Lines. Chem Eng Trans. 2017;56:1567-1572.

Yallapu MM, Chauhan N, Othman SF, Khalilzad-Sharghi V, Ebeling MC, Khan S, et al. Implications of protein corona on physico-chemical and biological properties of magnetic nanoparticles. Biomaterials. 2015;46:1-12.

Yuan S, Ding X, Cui Y, Wei K, Zheng Y, Liu Y. Cisplatin Preferentially Binds to Zinc Finger Proteins Containing C3H1 or C4 Motifs. Eur J Inorg Chem. 2016;2017(12):1778-1784.

Received for publication on 20th December 2020
Accepted for publication on 04th July 2021