Original Article

Probing the Biological evaluations of a new designed Palladium (II) complex using spectroscopic and theoretical approaches: Human Hemoglobin as a Target

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Abstract

Background: Previous studies reported that Palladium (Pd)(II) drug compounds showed significant anti-tumor activity in comparison with cis-platin. **Materials and Methods:** In this study, we investigated the biological evaluations of a designed Pd (II) complexes (bi pyridine ethyl dithiocarbamate palladium II nitrate) via its anti-proliferative effects on the alterations in the function and structure of human hemoglobin (Hb) at different temperatures of 25 and 37°. Also for further investigation, multi-spectroscopic methods such as fluorescence and the far-UV circular dichroism (CD) with hemoglobin target were assessed. **Results:** Fluorescence data showed the pure ability of Pd(II) complex to quench the intrinsic fluorescence of Hb. The binding constant, number of binding sites, and thermodynamic parameters at two temperatures were assessed and the results demonstrated the major possibility of occurring electrostatic and hydrophobic interactions in the Pd (II) complex–Hb interaction. For evaluating the change of secondary structure of Hb upon interaction with various concentrations of complex, far-UV CD spectra was applied and it was observed that in high dose of complex, significant changes occurred which is indicative of some side effects in overdosing of this complex. **Conclusion:** Our results suggested that using palladium complex as an anticancer agent might cause some disorders in structure and function of Hb as well as improve understanding of the side effects of newly designed metal anticancer drugs.

Keywords: Palladium complexes, Hemoglobin, Chemotherapy, Quenching.

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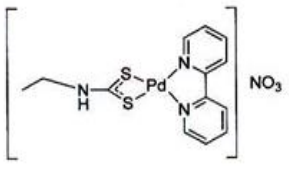
Introduction

After cardiovascular disorders, cancer is a serious cause of human death all over the world. One of best known method for cancer treatment is chemotherapy that uses metal compounds as anti-tumor agents. The use of metal-based complexes as therapeutic drugs dates back to many years ago (1). By easily losing electrons and making cationic form, the attraction of these opposing charges leads to a general tendency for metal ions to bind to and interact with biological molecules (2). But serious side effects have become obstacles in treatment when current compounds with anticancer activity are used, therefore it is necessary to search for new potential antitumor substances to improve treatment efficacy with smaller doses and less toxic drugs. The development of drugs that selectively eliminate cancer cells without harming normal ones also remains a considerable challenge (3). Based on the previous studies, it is proved that Pd (II) complexes have antitumor activity and cytotoxic effects on tumor cells (4). Palladium (and platinum) salts were shown to possess both histamine-releasing and histamine

binding properties. The latter effect might be one of the possible mechanisms of the antitumor effect of palladium as well as platinum salts, since histamine binding in tumor cells is suggested to suppress their proliferation (5). Many investigations demonstrate that hydrolysis of leaving ligands in palladium complexes is too rapid, 100 times faster than their related platinum analogues (2). Pt (II) complexes bind the thiol-containing enzymes of the renal and cause serious side effects on kidney, but palladium complexes with dithiol structural group have less kidney toxicity (6).

Hemoglobin is the most important oxygen transporter in red blood cells which carries oxygen from the lungs to the tissues in blood (7). This carrier protein consists of from two α-chains and two β-chains with141 (15126.1 Da) and 146 (15867.2 Da) amino acids, respectively (8). Some non-helical segments separate the α and β subunits which are made up by seven and eight helices, respectively (9). Also, Hb is classified as metallo-proteins for its heme-containing structure which join to each chain by a non-covalent interaction (10). Besides oxygen, hemoglobin is involved in the transport of other gases such as carbon dioxide (about 20-25% of the total). Hb also carries some regulatory molecules such as nitric oxide and heavy metal-based drugs for remedy aims (7).

These results lead us to investigate in more detail the reactivity of these derivatives towards vital proteins. In this study, a novel anticancer Pd(II) complex (bi pyridine ethyl dithiocarbamate palladium II nitrate) (Fig.1) was designed and binding properties and effects of this novel Pd(II) compounds on the structure and function of hemoglobin by different spectroscopic techniques (fluorescence and far UV circular dichroism (CD)) was investigated.



[Pd(bpy)(Et-dtc)]NO_3

Figure 1. The molecular structure of Pd(II) complex.

Methods

For extracting and purifying human Hb, previous well-established methods were applied. All other materials and reagents were of analytical grades, and solutions were made in double-distilled water.

Synthesis of Pd(II) complex. The solution of 1 mmol [Pd(phen)(H2O)2](NO3)2 in 20 ml was mixed with amyl-glycine hydrochloride (1 mmol) and NaHCO3 (0.17 gr, 2 mmol) dissolved in 9 ml distilled water at 50 ºC for 2 hours in dark. Then, light yellow solution was filtrated and concentrated to 15 ml at 35 ºC. The trace amount of turbidity formed was filtered and the clear yellow filtrate was further concentrated to about 5 ml at 35 ºC (very fine needle crystals of the complex not suitable for X-Ray crystallography were obtained by slow evaporation of this solution). The yellow crystals was filtered and washed with little amount of chilled double distilled water and then dried in vacuum oven.

[Pd(phen)(amylgly)]NO3 (M.W. =493.63 g/mol): The yield of the complex was 222 mg, %45; Elementary analysis calculated (found %) for C19H23N4O5Pd: C, 46.12 (46.16); H, 4.66 (4.8); N, 11.34 (11.22); IR (cm−1, solid): 3432 (w), 3051 (w), 2929 (w), 1625 (s), 1591, (s), 1511 (s) 1426 (s), 1354 (d), 1229 (s), 1139 (s), 1033 (s), 939 (s), 837 (s), 710 (s), 645 (s), 534 (s); 1H NMR (300 MHz, DMSO-d6, δ in ppm): 0.830 (t, 3H), 1.239 (m, 2H), 1.346 (m, 2H), 1.376 (m, 2H), 2.680 (m, 2H), 3.518 and 4.203 (d, 2H), 7.84 (s, NH), Aromatic protons: 9.340 (m, 2H), 9.130 (m, 2H), 8.750 (m, 2H), 8.100 (m, 2H).

0 (m, 2H), 8.750 (m, 2H), 8.100 (m, 2H).

Fluorescence measurements. For monitoring changes in intrinsic fluorescence intensity, Cary Eclipse Spectrofluorimeter (Varian Co., Australia) were carried out in the absence and presence of various concentrations of the Pd(II) complex (0, 3, 5, 7, 9, 11, 13, 15, 17 and 19 μM). The wavelength of excitation was established at 290 nm and emission spectra for all of the samples were recorded in the range of 300-500 nm at two temperatures of 25 and 37oC. All measurements were done by using a fluorescence cuvette with a 1 cm path length with 20 μM. Concentration of Hb.

Circular dichroism (CD) measurements. Alteration in CD spectra of Hb protein in the absence

and presence of various concentrations of a novel Pd(II) complexes (0, 2, 4 and 6 μM) were analyzed on an Aviv Spectropolarimeter (model 215 Proterion Corp., USA) in far-UV region (190–260 nm) using 1 mm path length at two temperatures of 25 and 37°C. The obtained data were given in molar ellipticity [θ] (deg cm2 dmol-1) based on a mean amino acid residue weight of 114 (MRW). The molar ellipticity at wavelength λ was fixed as [θ]λ= (100 × MRW × θobs/cl), where θobs is the monitored ellipticity in degrees at λ nm, c is the concentration of protein in mg ml-1, and l is the length of the light path in centimeters. For clarifying the secondary structure of the protein CD software of CDNN was applied based on the statistical method (11).

Results

Fluorescence Studies. One of the most powerful technique for investigating molecular interactions especially proteins is fluorescence spectroscopy (12). Since the intrinsic fluorescence of indol chromophores in Trp residues is extremely sensitive to their microenvironment, by studying the conformational alterations of proteins containing internal fluorophores, such as Trp residues, this method provides very useful information during the binding of ligands (13). Intrinsic fluorescence of Trp residue of Hb in the absence and presence of the different concentrations the Pd(II) complex were useful data for determining the mechanisms of drug binding to protein and its dynamic and structural functions (14). In tetrameric structure of Hb there are six Trp residues at all. Each αβ dimmer of Hb tetramer contains three Trp residues (α-14Trp, β-15 Trp and β-37 Trp) (15). The responsible intrinsic fluorophore is β-37 Trp residue at the α1β2 interface.

In this study, Hb solution was excited at 285 nm and the emission spectra were recorded between 300 and 450 nm at two temperatures of 25 and 37oC in the absence and presence of different concentrations of Pd(II) complex (Figure 2). As it is presented in Fig. 2 (only 25 oC is shown), the fluorescence intensity of Hb regularly decreased upon addition of Pd (II) complex. Since Fig. 2 represents, quenching of Hb with an increasing palladium/Hb ratio, interaction of the drug complex with Hb was occurred and refold the microenvironment of Trp residue(s), and also protein native structure.

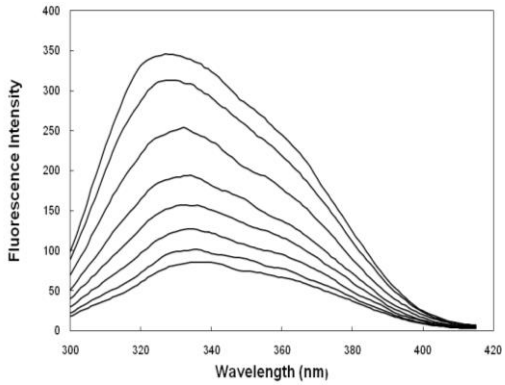


Figure 2. Fluorescence titration curve of Hb (5μM) in the absence and presence of various concentrations of Pd(II) complex in NaCl solution (5 mM) at 25˚C.

For predicting binding mechanisms of a drug to a protein, assessing the intrinsic fluorescence quenching of the studied protein provides very useful data. These data can be gathered by the calculating the accessibility of quenchers to the fluorophore groups of proteins (12). Fluorescence quenching usually proceeds via dynamic quenching or static quenching (16). In dynamic quenching, diffusive collisions between the fluorophores and the quencher in the excited state is usually occurred. Since it depends on diffusion effects, many of the coefficients and constants such as diffusion coefficients and bimolecular quenching constants increase with increasing temperatures. In contrast, the static mechanism shows different manner. In this mechanism, a ground-state complex between the fluorophores and the quencher is formed and therefore, a non-fluorescing complex is generated in static quenching which made a lower stability and the values of static quenching constants with higher temperature (17).

For investigating the quenching mechanism of the Pd (II) interaction with Hb, quenching experiments were carried out. For fluorescence quenching, the well-known Stern–Volmer equation usually is used to determine the decrease in intensity (18):

$$= 1 + Ksv[Q] = 1 + kq\tau o[Q] \quad (1)$$

where Fo and F are the fluorescence intensities in the absence and presence of quencher (Pd(II) complex) respectively, [Q] is the concentration of the

Table1. Different parameter of Hb interaction with Pt(II) complex at different temperatures of 25 and 37 ˚C

Temperature (°C)	KSV (×106 M-1)	kq (×10-12 M-1S-1)	Kb (×10-4 M-1)	n	ΔG0 (kJ/mol)	ΔH0 (kJ/mol)	TΔS0 (KJ/mol.K)
25	0.21±0.001	1.4±0.07	0.25	1 ±0.3	-30.7±1	-22 ±6	-11.3
37	0.23±0.0008	0.7±0.03	6.3	1 ±0.3	-40.7±0.2		

quencher, Ksv is the Stern–Volmer quenching constant, kq is the quenching rate constant of protein, and τ0 is the average life time of the protein without any quencher (Pd(II) complex) (19).

The experimental data were analyzed using Equation (1), and the results were presented in Figures 3 and Tables 1. The results of Fig. 3a and Table 1 show that the Stern–Volmer plot is linear and the values of Stern-Volmer quenching constants, KSV, is not increased by increasing the temperature, indicating the presence of static quenching of fluorescence in Hb in Pd(II) complex.

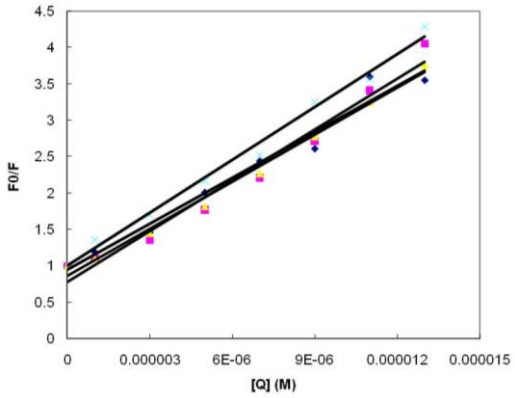


Figure 3a. Stern-Volmer plots of Pd(II) complex and Hb interaction in NaCl solution (5 mM) at different temperatures of 25 (●) and 37˚C (○).

Binding Studies. As mentioned above, fluorescence experiments demonstrate that Trp fluorescence quenching during Pd(II) interactions with Hb follows a static quenching type. Therefore, according to the literature, estimating the binding parameters of the Pd(II) complex to protein can be possible by following equation (18):

$$= \log K + n \log [Q] \qquad (2)$$

Where K is the binding constant and n is the binding number (18). Table 1 present the calculated values of n and K. According to Eq. (2), the binding number can be estimated from the slope of the static quenching plot of log [(F0-F)/F] versus log [Q] (18). All resulted data at different temperatures of 25 and37oC for the drug complex are also summarized in Table 1. By concerning these Figure and Table, the value of n is close to 1 for the Pd (II) complex and hence there is one binding site in Hb structure for each of the Pd(II) complexes. Finally, concerning the tables and figures, estimated binding constants for the drug compounds at 25 and37 oC reveal that the complex formation between Pd(II) and Hb is affected by heat. In other words, there is an exothermic reaction in both reactions (Fig. 3b and Table 1).

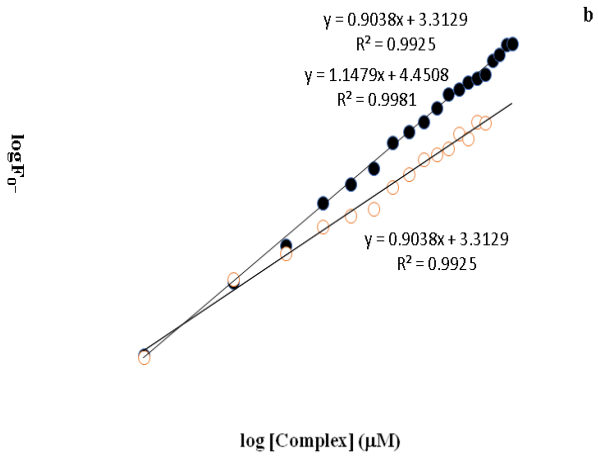


Figure 3b. The plot of log (F0-F/F) vs. log [Pd] resulted from fluorescence quenching data at different temperatures of 25 (●) and 37˚C (○).

Determination of thermodynamic parameters. As mentioned above, a thermodynamic process is responsible for the formation of the complex. hence, dependency on heat was analyzed for better characterizing the forces acting between ligands and protein (20). Essentially, there are four types of non-covalent interactions stabilizing ligand-protein connections: hydrophobic interactions, van der Waals

forces, hydrogen bonds and electrostatic forces (21). Enthalpy change (ΔHo) and entropy change (ΔSo) of binding reactions are two main criteria confirming binding modes in thermodynamic parameters (12). Using the van't Hoff equation for estimating thermodynamic parameters is a common way for terms that the alteration in interaction enthalpy and entropy does not vary significantly over the temperature range studied (16).

$$\ln K = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} \quad (3)$$

where K is the binding constant obtained from Eq. (2) at the corresponding temperature (T), and R is the gas constant (22). Also, the free energy change (ΔG°) is determined from the following relationship (23).

$$\Delta G^{\circ} = \Delta H^{o} - T\Delta S^{o} = -RT \ln K \quad (4)$$

According to previous studies, (24) during protein association processes, the sign and magnitude of thermodynamic parameters with different individual types of interactions that may occurred, from which it can usually be concluded: (a) ΔH° > 0 and ΔS°> 0, hydrophobic forces; (b) ΔH° < 0 and ΔS° < 0, van der Waals forces and hydrogen bonds; (c) ΔH° <0 and ΔS° > 0, electrostatic interactions (25). The estimated values of thermodynamic parameters of the palladium complex are summarized in Table 1. In this manuscript, the negative sign for the values of the ΔG° for the Pd(II) complexe during interaction with Hb is indicative of spontaneous interactions (26). Also the negative values of ΔH° and the positive value of ΔSo for binding of complexes to Hb are usually taken as an evidence for electrostatic interactions (27). These kinds of interactions usually accompanied with surface hydrophobicity increase and may be confirmed that electrostatic and hydrophobic interaction might be responsible for the occurrence of interactions between the drug compound and Hb.

CD Results. Another ideal technique for studying conformational changes in protein secondary and tertiary structures is Circular Dichroism (CD). Some experimental conditions such as ligand-protein binding can causes CD occurrence. To characterizing the secondary structure of proteins due to peptide bond absorption, the far-UV-CD spectra is a common way (28). In this study, the structural changes of Hb in the absence and presence of various concentrations of the Pd (II) complex at several temperatures of 25 and 37oC were recorded and are presented in Figs. 4 and 5. The contents of secondary structure elements in Hb in the absence and presence of various concentrations of the Pd (II) complex at different temperatures of 25 and 37oC were monitored and all resulted data are shown in tables 2 and 3. Changes in Hb secondary structure demonstrate the degree of structural stability. Protein structural stabilization in the presence of ligand is observed when an increase in β -sheet and α -helix contents and a decrease in random coil content occur. Conversely, decrease in protein structural stability is monitored by a decrease in b-sheet and a-helix contents and an increase in random coil content in the

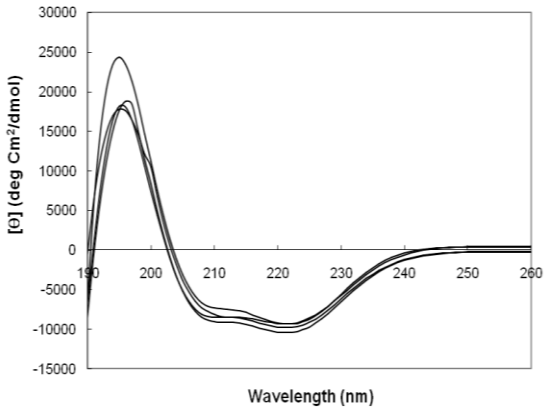


Figure 4. Far-UV CD spectra of Hb (10.2 µM) in the absence and the presence of different concentrations of Pt(II) complex 93.3, 186.6, 266.6 µM (from down to up) in NaCl solution (5 mM) at 25˚C.

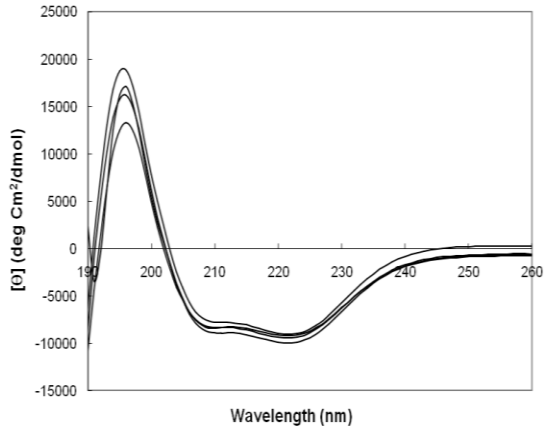


Figure 5. Far-UV CD spectra of Hb (10.2 µM) in the absence and the presence of different concentrations of Pt(II) complex 93.3, 186.6, 266.6 µM (from down to up) in NaCl solution (5 mM) at 37˚C.

presence of ligand (28). All resulted data summarized in Fig. 5 and Table 2-3. Concerning all these data imply that Pd (II) complexes /Hb interaction causes a decrease in protein stability.

Table2. Changes in the secondary structure of Hb upon interaction with the Pd(II) complex at 25°C

Pd (II) complex (μM)	%α-Helix	%β-Sheet	%Random coil
0	57.80	21.80	20.40
2	56.10	21.50	22.40
4	51.50	23.80	24.70
6	53.70	22.90	23.40

Table3. Changes in the secondary structure of Hb upon interaction with the Pd(II) complex at 37°C

Pd (II) complex (μM)	%α-Helix	%β-Sheet	%Random coil
0	51.60	22.40	24
2	51.30	23.60	25.10
4	50.10	25.20	24.70
6	45.50	26.60	27.90

Discussion

Fluorescence measurements implied that static quenching is dominated mechanism in the quenching. Furthermore, from the estimated thermodynamic parameters, there is one binding site for the drug compound in protein structure. ΔG°, ΔHo and ΔSo values were determined at four temperatures of 25 and 37°C for the drug compound and resulted data reveal that electrostatic and hydrophobic forces proceed interaction between the Pd(II) complex and Hb. Also, negative sign for the Gibbs free energy value during the binding the Pd (II) complex to protein was indicative of a spontaneous interaction. CD spectroscopy monitoring implied that the drug-protein interaction caused an alteration in α-helix contents of the hemoglobin at experimented temperatures and also significant changes in tertiary structure of the Hb.

Conclusion

The development of a sufficient Pd (II) anticancer compound involves particular requisites: stabilization by a strongly coordinated ligand coupled to an exact choice of leaving group(s), in order to ensure the in vivo structural integrity of the compound for a long enough period to enable it to perform its therapeutic action (29). These results provide useful information for designing effective anti-cancer compounds in order to find a trustworthy way for the treatment of cancer.

Conflicts of Interest

There is no conflict of interest among authors.

Acknowledgment

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