Heme Releasing from Human Hemoglobin upon Interaction with a New Synthesized Complex of 1,10-Phenanthroline-n-butyl Dithiocarbamato Pd(II) Nitrate

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In the present study, we investigated the effect of a new anticancer Pd(II) complex, 1,10-phenanthroline-n-butyl dithiocarbamato Pd(II) nitrate, on the heme releasing from human hemoglobin (Hb) as well as alterations in the structure and function of Hb using different spectroscopic methods of UV-Vis, fluorescence and circular dichroism (CD)at two temperatures of 25 and 37 °C. Fluorescence data revealed that the Pd (II) complex is able to quench the intrinsic fluorescence of Hb. The binding constant, number of binding sites and thermodynamic parameters at two temperatures were calculated. The values of $\Delta H°, \Delta S°,$ and $\Delta G°$ indicated that the van der Waals force or hydrogen bond interactions might play a major role in the interaction of complex with Hb. The far-UV-CD studies displayed that the regular secondary structure of Hb had no significant changes. To evaluate the functional changes of Hb *via* destruction of the heme structure, fluorescence studies were performed at excitation wavelengths of 321 nm and 460 nm with emission wavelengths of 465 nm and 525 nm, respectively. The results demonstrated that two fluorescent heme degradation products are found during the interaction of Pd(II) complex with Hb. The results of thermal behavior of Hb studied at 415 nm confirmed the heme degradation, which referred to decrease in the hemoglobin stability in the presence of Pd(II) complex. Also, the cytotoxic effects and anti-tumor activity of the complex against human breast cell line, T47D were carried out using MTT assay. The Cc_{50}value obtained after different incubation times of 24 and 48 h. The finding related to structural and functional changes of Hb induced by Pd(II) complex may be important to improve understanding of side effects of new designed metal anti-cancer drugs undergoing.

Keywords: Hemoglobin, Palladium(II) complex, Heme degradation, Fluorescence, Cytotoxicity

INTRODUCTION

Hemoglobin (Hb) is one of the most effective proteins in the blood that carries oxygen from the lungs to the tissues [1]. Hb is a tetramer protein, made up of two α-chains, each containing 141 amino acids (15126.4 Da) and two β-chains, each containing 146 amino acids (15867.2 Da) [2]. The α- and β-subunits contain seven and eight helices, respectively, joined by non-helical segments [3]. The heme group is linked to the each chain by noncovalent interaction [4]. Hb is an allosteric protein, this means that the binding an effector molecule to one of the subunits is affected by its interactions with the other subunits that it is in equilibrium between two structures: the deoxy or T structure with low oxygen affinity, and the oxy or R structure with high oxygen affinity [5,6].

The interaction of ligands with Hb may be caused by changing in the tertiary structure of this protein and so, equilibrium between two structures, R and T, are shifted to the right or left, resulting in the tendency of Hb for oxygen decreases or increases [7]. Some drugs can bind to Hb such as heavy metals based drugs. Platinum-based drugs were

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discovered for the first time in 1969 by Rosenberg's [8]. These drugs have played a key role among the metal-based anticancer agents [9]. Examples of anticancer drugs are cisplatin, oxaliplatin, nedaplatin and carboplatin that have been used in the treatment of a variety of human cancers [10], such as ovarian, testicular, lung, urinary bladder, head, and neck cancer [11]. Pt containing anticancer drugs by covalently binding to quinine of DNA causing damage to the DNA double helix and the leading to apoptosis in cancer cells [12]. However, Pt-containing drugs have several side effects, such as nephrotoxicity, neurotoxicity, emetogensise, vomiting and anemia [13]. These drugs are introduced intravenously, therefore, they react with blood plasma proteins, such as serum albumin and Hb [14]. The formation protein-anticancer drugs complex are the drug's side effects, too [15]. Since platinum and palladium are chemically very similar, it was expected that the palladium complexes also display anti-cancer activities [16]. So, it can be expected that these compounds to have lower toxicity than that of platinum complexes. Recently, Pd-containing drugs have been attention to the treatment of a variety of human cancers [17].

In our previous studies, a new anticancer Pd(II) complex, (1,10-phenanthroline butyl dithiocarbamato palladium(II) nitrate, [Pd (Bu-dtc) (phen)]NO$_3$) (Scheme 1) was designed and its interaction with DNA was investigated [18]. We showed that the complex presumably intercalates in DNA and induces apoptosis in cancer cells. In the present research, we have studied the effects of that new designed anticancer Pd(II) complex on the structure and function of blood protein of Hb by fluorescence, UV-Vis and circular dichroism (CD) spectroscopic techniques at two temperatures of room (25 °C) and physiologic (37 °C). Finally, the cytotoxic and antiproliferative activity of the complex have been checked against a model cancer cell line of T47D after different incubation times of 24 and 48 h.

EXPERIMENTAL

Materials

Human Hb was extracted and purified by well-established methods [2]. Pd(II) complex was synthesized in our laboratory using previous procedures [18]. All other materials and reagents were of analytical grade and
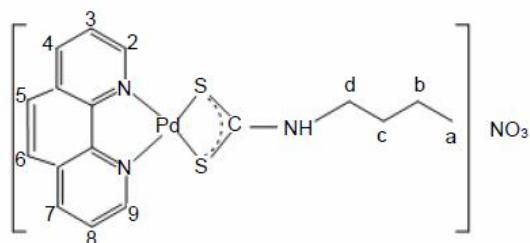
Scheme 1. The molecular structure of 1,10-phenanthroline butyl dithiocarbamato palladium(II) nitrate.

solutions were made using double-distilled water. Since Pd(II) complex does not dissolve in any buffers then we had to dissolve it in NaCl (5 mM) solution, adjusted to pH 7.

Methods

Fluorescence measurements. Fluorescence intensity measurements were carried out using a Cary Eclipsespectrofluorimeter (Varian Co., Australia). The excitation wavelength was adjusted at 285 nm and the emission spectra were recorded for all of the samples in the range of 290-450 nm at 25 and 37 °C, using a 1 cm path length fluorescence cuvette. Fluorescence spectra of the Hb (6 μM) were measured in the presence of different concentrations of Pd(II) complex from 0 up to 127 μM.

Heme degradation fluorescent. Heme degradation fluorescent emission spectra of the Hb (6 μM) in the presence of different concentrations of Pd(II) complex from 0 up to 90 μM at two temperatures of 25 and 37 °C were also scanned from 400 to 600 nm at an excitation wavelengths of 321 and 460 nm [19,20].

Circular dichroism (CD) measurements. CD spectra were recorded on an Aviv Spectropolarimeter (model 215 Proterion Corp., USA). Changes in the secondary structure of Hb was monitored in the far-UV region (190-260 nm) using 1mm path length at 25 and 37 °C in the absence and presence of different concentrations of Pd(II) complex (0, 47.6, 117.6 and 208 μM).The protein concentration in the experiments for far-UV region was 10.8 μM. The results were expressed in molar ellipticity [θ] (° cm^2 dmol^{-1}) based on a mean amino acid residue weight of 114 (MRW). The molar ellipticity was determined as $[θ]_λ = (100 × MRW × θ_{obs}/cl)$, where $θ_{obs}$ is the observed ellipticity in degrees at a

given wavelength, c is the protein concentration in mg ml^{-1} and, l is the length of the light path in cm. The CD software was used to predict the secondary structure of the protein according to the statistical method [21,22].

Thermal denaturation measurements. The thermal behavior of Hb (16 μM) at various concentrations of Pd(II) complex (0, 14.8 and 37 μM) was studied at 278 and 415 nm in the temperature range of 25-95 °C by Cary spectrophotometer (Bio-model 100), with jacketed cell holders.

Cell line. Human breast cancer cell line, T47D was obtained from the cell bank of Pasteur Institute of Iran. These cells were maintained in RPMI 1640 medium supplemented with 10% FBS in a humidified incubator (37°C and 5% CO_2).

Cell culture. The cells were grown in RPMI medium supplemented with L-glutamine (2 mM), streptomycin and penicillin (5 μg ml^{-1}) and 10% heat-inactivated fetal bovine serum, at 37 °C under a 5% CO_2/95% air atmosphere.

Cell proliferation assay. The growth inhibitory effect of Pd(II) complex towards the cells was measured by means of MTT assay. The cleavage and conversion of the soluble yellowish MTT to the insoluble purple formazan by active mitochondrial dehydrogenase of living cells were used to develop an assay system alternative to other assays for measurement of cell proliferation. Cells were plated in 96-well tissue culture dishes and after 24 h, varying concentrations of the sterilized complex (0-680 μM) were added and incubated for 24 and 48 h. At the end of four hours, incubations 25 μl of MTT solution (5 mg ml^{-1} in PBS) was added to each well containing fresh and cultured medium. At the end, the insoluble formazan produced was dissolved in a solution containing 10% SDS and 50% DMF (Left for 2 h at 37 °C in dark conditions), and optical density (OD) was read against the reagent blank with a multi well scanning spectrophotometer (ELISA reader, Model Expert 96, Asys Hitchech, Austria) at a wavelength of 570 nm. Absorbance is a function of concentration of converted dye. The OD value of study groups was divided by the OD value of untreated control and presented as percentage of control (as 100%).

Statistical analysis. Results were analyzed for statistical significance using two-tailed Student's *t*-test. Changes were considered significant at $p < 0.05$.

RESULT AND DISCUSSION

Fluorescence Studies

Fluorescence spectroscopy is a powerful tool to study molecular interactions involving the interaction between ligand and protein [23]. The intrinsic fluorescence of tryptophan residues and intrinsic fluorescence quenching of Hb in the presence of the ligand can help predict the binding mechanisms of drug to Hb [24]. Hb contains three Trp residues in each αβ dimer, for a total of six in the tetramer: two α-14 Trp, two β-15 Trp, and two β-37 Trp [25]. Hb intrinsic fluorescence is primarily originates from the β-37 Trp at the $\alpha_1\beta_2$ interface and is sensitive to the R-T transition [26]. The fluorescence emission of β-37 Trp and R-T transition play an important role in depicting the changes that be revealed in the quaternary structure of Hb upon ligand binding, which these changes are reflected in the fluorescence intensity [27].

Figure 1 shows the fluorescence emission spectra of Hb at different fixed concentration of Pd(II) complex with $\lambda_{ex} =$ 285 nm at two temperatures of 25 and 37 °C. Results represent that the emission of Hb gradually decreases with increasing the Pd(II) complex concentration. This result shows that the Pd(II) complex is able to quench the intrinsic fluorescence of Hb and the interaction between Pd(II) complex and Hb is occurred.

Generally, the fluorescence quenching is classified as dynamic quenching and static quenching. Dynamic quenching depends upon diffusion. Diffusion coefficients increase with increasing temperature, so the bimolecular quenching constants increase in dynamic quenching. While the static quenching constants decrease with increasing temperature in a static quenching. The fluorescence quenching data for Hb were analyzed using to Stern-Volmer equation, Eq. (1), [28].

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \tag{1}$$

Where F_0 and F are the fluorescence intensities of Hb in the absence and presence of different concentration of Pd(II) complex, respectively. K_{SV} is the Stern-Volmer dynamic quenching constant, k_q is the quenching rate constant of Hb, and τ_0 is the average life time of the molecule without any
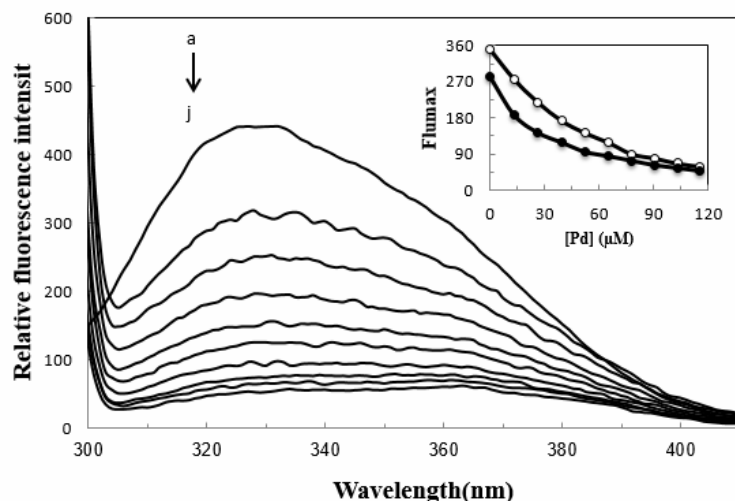
Fig. 1. Fluorescence titration curve of Hb (6 μM) with the Pd(II) complex in 5 mM NaCl solution at 25 ℃.

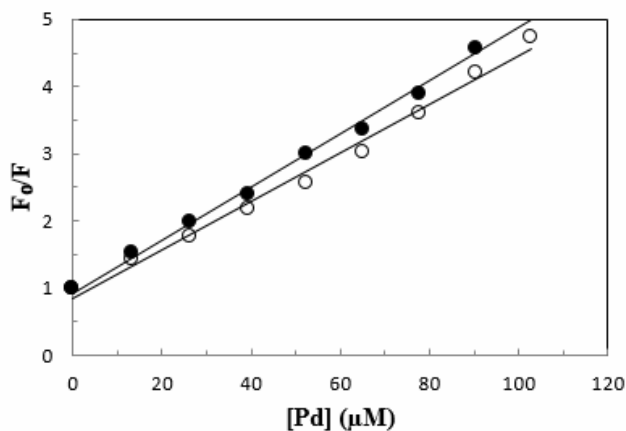**Fig. 2.** Stern-Volmer plots for the interaction of Pd(II) complex and Hb in 5 mM NaCl solution at 25 (○) and 37 ℃ (●).

quencher (Pd(II) complex). Data were fitted by Eq. (1) and shown in Fig. 2. The values of K_{SV} and k_q were calculated from the slope of Stern-Volmer plots and were presented in Table 1. The date showed that Stern-Volmer quenching constant, K_{SV}, is directly correlated with temperature and increases with increasing of temperature from 25 to 37 ℃. Also, the values of k_q are larger than diffusion controlled quenching constant value of the order of ~1 × 10^{10} M^{-1} s^{-1} [29]. It suggests that quenching mechanism of fluorescence of Hb by Pd(II) complexes is initiated by a dynamic
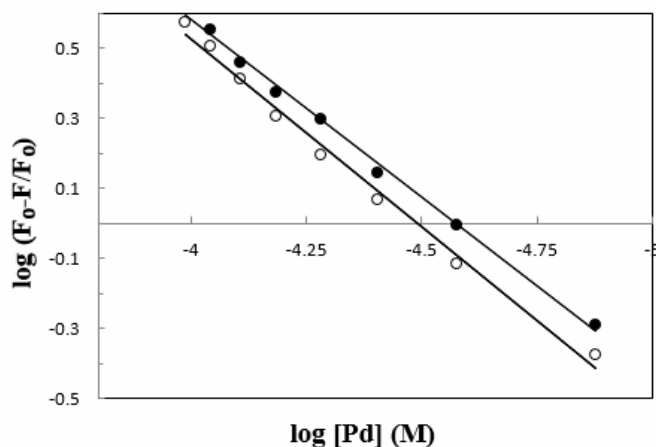
collision and compound formation.

Binding Study

To understand and obtain the binding and thermodynamic parameters of the interaction of Pd(II) complex and Hb at two temperatures of 25 and 37 °C the plot of log(F_0 - F)/F vs. log[Q] according to the Eq. (2) [30,31] was prepared:

$$\log(\frac{F_0 - F}{F}) = n\log[Q] + \log K_b \qquad (2)$$

Table 1. Various Parameters of Hb upon Interaction with Pd(II) Complex at Two Different Temperatures of 25 and 37 °C

T (°C)	K_{SV} ($\times 10^4$ M^{-1})	k_q ($\times 10^{12}$ M^{-1} S^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})	K_b ($\times 10^5$ M^{-1})	n
25	3.6	3.6		-27.5		6.6	1.0
			-27.8		-1		
37	3.9	3.9		-27.6		4.4	1.0

Fig. 3. The plot of log(F_0 - F)/F *vs.* log[Pd] resulted in the fluorescence quenching data at 25 (○) and 37 ℃ (●).

Where n is the number of binding sites and K_b is the binding constant. Figure 3 and Table 1 show the parameters obtained from the interaction of Pd(II) complex and Hb at two temperatures of 25 and 37 °C. There is one binding site for Hb to bind to Pd(II) complex at both temperatures ($n =$ 1). The value of binding constant (reported in Table 1) decreases with increasing the temperature, indicating the formation of an unstable complex between Hb and Pd(II) complex that might be disassociated with the rising temperature [32].

Determination of Thermodynamic Parameters

The interaction force between a drug and a macromolecule can be included hydrogen bond, electrostatic force, van der Waals force, and hydrophobic interaction force, *etc.* [33]. To clarify the type of binding force for the interaction between Hb and Pd(II) complex, thermodynamic parameters of enthalpy change ($\Delta H°$) and

entropy change ($\Delta S°$) were determined using the van't Hoff equation (Eq. (3)) [34]:

$$\ln K_b = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \tag{3}$$

T and R are the temperature and gas constant, respectively. In addition, the standard Gibbs free energy ($\Delta G°$) can be calculated by Eq. (4) [35]:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \ln K_b \tag{4}$$

All values of $\Delta H°$, $\Delta S°$ and $\Delta G°$ have been summarized in Table 1. The values of $\Delta H°$ and $\Delta S°$ of the binding reaction between Pd(II) complex and Hb are found to be negative. The $\Delta G°$ value is also negative. The negative value of $\Delta G°$ indicates the spontaneity of the binding of Pd(II) complex to Hb [36]. Based on previous studies, Yang *et al.* associated

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the sign and magnitude of thermodynamic parameters with various individual kinds of interactions that may take place during protein association processes, from which it can easily be concluded: (a) $\Delta H° > 0$ and $\Delta S° > 0$, hydrophobic forces; (b) $\Delta H° < 0$ and $\Delta S° < 0$, van der Waals forces and hydrogen bonds; (c) $\Delta H° < 0$ and $\Delta S° > 0$, electrostatic interactions [37]. Then, the negative values of $\Delta H°$ and $\Delta S°$ show that the van der Waals force or hydrogen bond interactions might play a major role in the interaction of Pd(II) complex with Hb [38].

Heme Degradation Study

Hb contains around 80% of total heme in the body. Heme links tightly to the hydrophobic pocket of the globin chain by covalent bonds between irons in heme and N of histidines and it mediates the normal physiological role of Hb in oxygen transport [39]. Ferrous state (Fe^{2+}) in the prosthetic group of heme in Hb combines with molecular oxygen [40]. When the heme is destroyed, the ferrous form (Fe^{2+}) is converted to the ferric form (Fe^{3+}) and therefore Hb is unable to carry oxygen as before. Hemes of Hb are non-fluorescent; however, releasing the iron or heme degradation can produce fluorescent products. Then, the formation of fluorescent products occurs due to releasing the iron or heme degradation of Hb [41,42]. During the interaction of Hb with Pd(II) complex, two fluorescent bands have been identified. One band revealed a maximum excitation wavelength at 321 nm with an emission wavelength of 465 nm, other band revealed a maximum excitation wavelength at 460 nm with an emission wavelength of 525 nm. As shown in Fig. 4, the fluorescence intensity for these two regions increases with increasing the
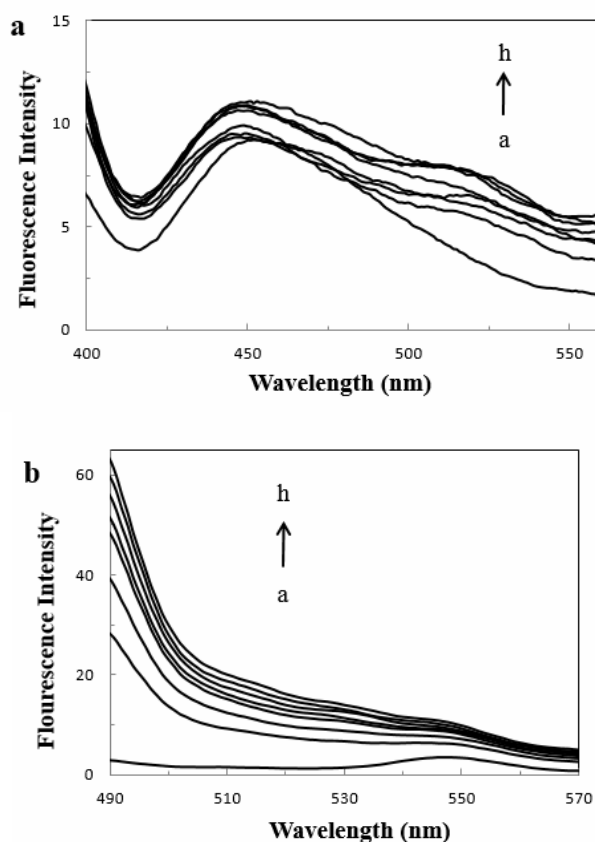

Fig. 4. Formation of fluorescent bands during the interaction of Hb (6 μM) with Pd(II) complex in 5 mM NaCl solution. (a) The emission spectra from 400 to 560 nm at excitation wavelength of 321 nm, and (b) the emission spectra from 490 to 570 nm at excitation wavelength of 460 nm.

concentration of Pd(II) complex (from 0 up to 90 μM),indicating the formation of two fluorescent heme degradation products; releasing the iron takes places at both temperatures of 25 and 37 ºC.

CD Studies of Pd(II) Complex Interaction with Hb

To obtain more information on the structural changes of Hb, due to the binding of Pd(II) complex, the CD spectra of Hb in the absence and presence of different concentrations of Pd(II) complex at two temperatures of 25 and 37 °C were studied and shown in Fig. 5. The CD spectrum of Hb exhibits two characteristic peaks of negative ellipticity at 208 nm and 222 nm, refering to α-helical secondary structure of the protein [43]. Figures 5 and Table 2 show that with increasing the concentration of Pd(II) complex at

25 °C (the data has not been shown at 37 °C), the α-helical content of the protein decreases whereas, the content of β-structures increases, which is indicative of alterations in a regular secondary structure of the protein upon interaction with complex.

Thermal Denaturation Studies

The thermal behavior of Hb was studied in the absence and presence of Pd(II) complex at a temperature range of 25-95 °C, at two wavelengths of 278 nm and 415 nm. Thermal denaturation of this biomolecule was examined by UV-Vis Spectroscopy [44] to obtain the T_m (the midpoint temperature of thermal denaturation) values of Hb at different concentrations of Pd(II) complex. The thermal behavior of Hb in the presence of Pd(II) complex has been
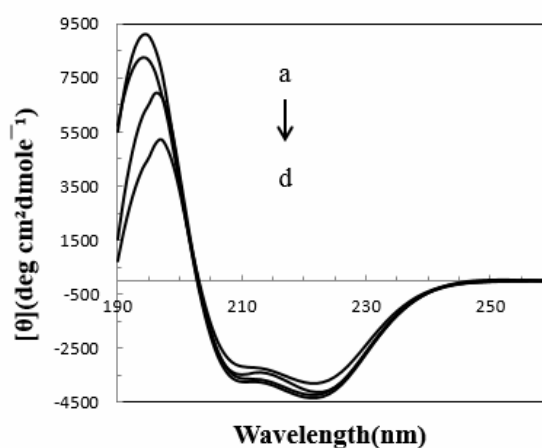

Fig. 5. Far-UV CD spectra of 10.8 μM Hb measured in the absence (a) and the presence of different concentrations of Pd(II) complex 47.6 (b), 117.6 (c) and 208 μM (d) in 5 mM NaCl solution at temperature of 25 °C.

Table 2. Changes in the Secondary Structure of Hb upon Interaction with the Pd(II) Complex at Temperature of 25 °C

[Pd] (μM)	%α-helix	%β-sheet	%Random coil
0	37.6	31.1	31.2
23.8	36.1	31.4	32.3
58.8	35.3	32.1	32.4
104	32.4	34.5	32.9

shown in Fig. 6. Determination of the standard Gibbs free energy of denaturation ($\Delta G°$), as a measure of conformational stability of Hb and T_m values, are based on the two-state theory as follows [45]:

$$Native\ (N) \Leftrightarrow Denatured\ (D) \quad (5)$$
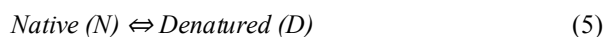
According to the two-state theory applied to monitoring changes in the absorbance, we can obtain the fraction of the denatured protein (F_d) and equilibrium constant (K) based on the following Eq. (6) [46]:

$$F_d = \frac{Y_N - Y_{obs}}{Y_N - Y_D} \quad (6)$$

$$K = \frac{F_d}{1 - F_d} = \frac{Y_N - Y_{obs}}{Y_N - Y_D} \quad (7)$$

Where Y_{obs} is the observed variable parameter such as ellipticity or absorbance, Y_N and Y_D are the values of ellipticity or absorbance of native and denatured forms, respectively. Then the $\Delta G°$ value for protein denaturation is given by Eq. (4):
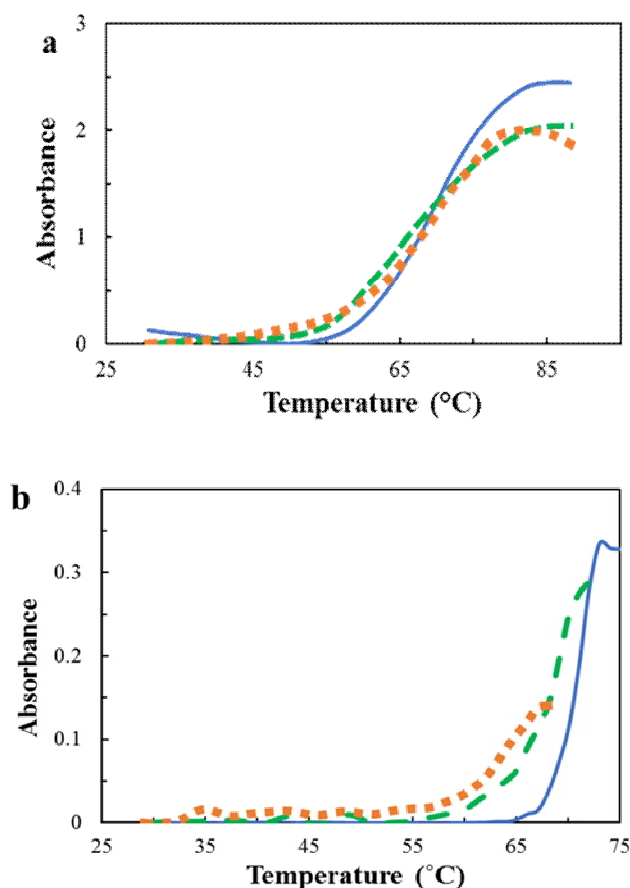
$$\Delta G^0 = -RT \ln K \quad (4)$$

Fig. 6. Thermal denaturation curves of Hb (16 μM) indifferent concentrations of complex 0 (——), 15 (- - -) and 37 (▪ ▪ ▪) μM at a temperature range of 25-95 °C at two wavelengths of (a) 278 nm and (b) 415 nm in 5 mM NaCl solution.

The plots of $\Delta G°$ *vs*. T (°C) at 278 nm (a) and 415 nm (b) are presented in Fig. 7, and $\Delta G°_{25°C}$ can be obtained from the Y-intercepts of the curve in Fig. 7. The values of $\Delta G°_{25°C}$ are the most valuable parameters of protein conformational stability in the process of thermal denaturation. The values of $\Delta G°_{25°C}$ and T_m are represented in Table 3 at different concentrations of Pd (II) complex. As seen in Fig. 7 and according to the results given in Table 3 in the presence of Pd(II) complex at different fixed concentrations, both values of $\Delta G°_{25°C}$ and T_m are reduced, referring to the decrease of Hb stability in the presence of complex. The reduction stability at 415 nm in the presence of Pd(II) complex is indicative of heme degradation and the release of iron of Hb [47].

Cytotoxicity Results

The new synthesized Pd(II) complex was studied for its anti-tumor activity on human breast cell line, T47D. In this study, different concentrations of Pd(II) complex (0-680 μM) were used to culture the tumor cell lines for 24 and 48 h. The 50% cytotoxic concentrations (Cc_{50}) value of Pd(II) complex was determined from Fig. 8 and shows that the Cc_{50} values of complex significantly have been decreased after higher incubation times. As shown in Fig. 8, the Cc_{50} values of Pd(II) complex were calculated 400 μM that the Cc_{50} value of Pd(II) complex obtained after incubation 48 h. Also, cell growing after higher incubation times reduced in different concentrations of Pd(II) complex. Then, it is clear that the Pd(II) complex shows a dose and time-response
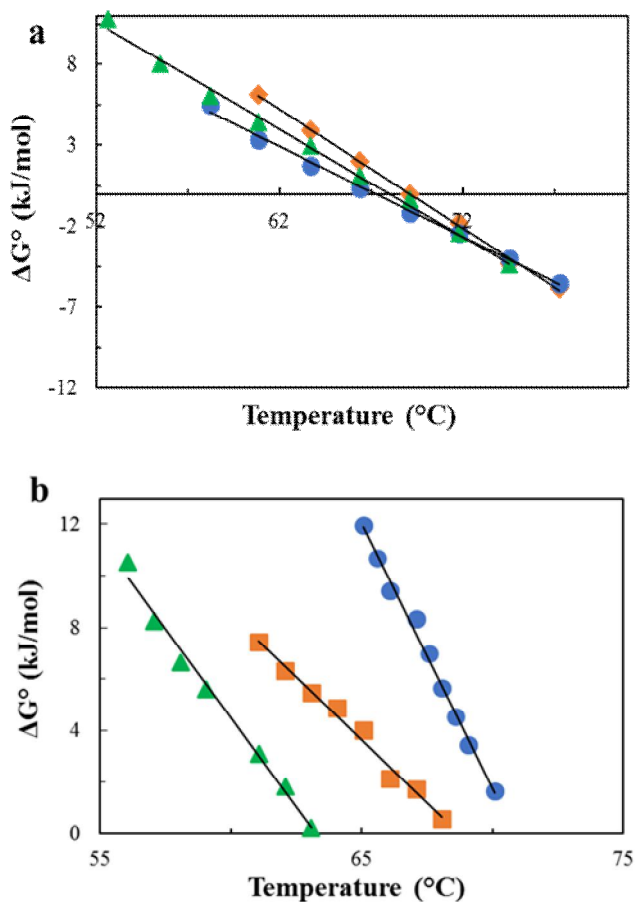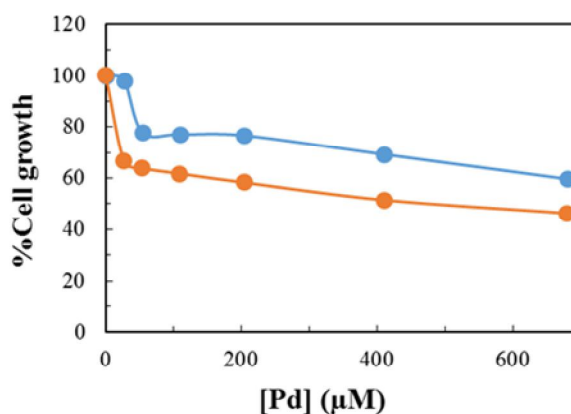

Fig. 7. The molar Gibbs free energies of unfolding of Hb in the presence of 0 (■), 15 (▶) and 37 (●) μM at a temperature range of 25-95 °C at two wavelengths of (a) 278 nm and (b) 415 nm.

Table 3. $\Delta G°_{25°C}$ and T_m Values of Hb Denaturation upon Interaction with the Pd(II) Complex at Wavelengths of 278 nm and 415 nm

[Pd] (μM)	λ	T_m (°C)	$\Delta G°_{25°C}$ (kJ mol^{-1})
0	278	75.0	32.4
	415	76.0	64.6
15	278	73.0	28.5
	415	73.4	52.8
37	278	70.7	23.6
	415	71.0	42.6

Fig. 8. The growth suppression activity of the Pd(II) complex on T47D cell line using MTT assay after different incubation times of 24 h (●) and 48 h (●).

suppression on growing of T47D breast cancer cell lines.

CONCLUSIONS

Our finding shows that the reaction between Hb and Pd(II) complex, 1,10-phenanthroline-n-butyl dithiocarbamato Pd(II) nitrate, is associated with structural and functional changes of Hb. These changes coincide with the quenching of the intrinsic fluorescence of Hb, releasing the fluorescence heme degradation products and decreasing the thermal stability of Hb. Our results also revealed that the Pd(II) complex has growth inhibitory activity against

T47D.Since the conformational changes in the one of the vital proteins in the blood due to interaction with the complex studied, be evaluated as side effects of this compound, therefore, the results provide useful information to understanding the mechanisms of action of these synthesized compounds and to identifying and introducing compounds with low toxicity and side effect.

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are highly appreciated.

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