

Design, Synthesis, and Biological Evaluation of a New Palladium(II) Complex: β -Lactoglobulin and K562 as Targets

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A water-soluble Pd(II) complex (2,2'-bipyridinglycinato Pd(II) nitrate) has been synthesized and characterized. The effect of synthesized complex on the carrier model protein structure and cell proliferation was investigated. Whey carrier protein β -lactoglobulin-B (BLG-B) and chronic myelogenous leukemia cell line K562 were the targets. Fluorescence and CD instruments were used to assess effect of the complex on the protein structure at different temperatures. Growth inhibitory and apoptotic effect of the Pd(II) complex toward the cancer cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. This complex exhibited potent cytotoxic properties against chronic myelogenous leukemia cell line K562. The cells showed different sensitivity to complex. Cytotoxic studies shown that Pd(II) complex induced apoptosis of K562 cells in a concentration and time dependent manner. Then, it might be concluded that Pd(II) complex is a promising antiproliferative agent and should execute its biological effects by inducing apoptosis. Results of fluorescence studies revealed that Pd(II) complex can quench the intrinsic fluorescence emission of the protein at different temperatures. The far- and near-UV CD studies displayed that the Pd(II) complex induces changes in the secondary and tertiary structures of BLG-B at different temperatures. The biological significance of this work is evident since BLG serves as a carrier molecule for several antitumor compounds. Therefore, the interaction of the Pd(II) complex (with antitumor activity) can provide useful information to better design metal anticancer complexes with fewer side effects. (Abbreviations: BLG-B, β -lactoglobulin-B; CD, circular dichroism; ΔH° , enthalpy; ΔS° , entropy; ΔG° , Gibbs free energy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Introduction

β -Lactoglobulin (BLG) is a small globular protein that forms the major component of ruminant milk whey,¹ composed of 162 amino acid residues and two disulfide bonds (Cys66-Cys160 and Cys106-Cys119). The BLG structure and unfolding transitions have been well characterized by various physicochemical studies.² This small globular protein has a three-dimensional structure consisting of eight strands of antiparallel β -sheet twisted into a cone-shaped barrel that constitutes a hydrophobic pocket.^{3–6} BLG is a well-studied member of the lipocalin family. The physiological function of BLG is tentatively considered to be the binding and transportation of small hydrophobic ligands, such as retinal and fatty acids.⁷ In 1955, Townend⁸ found that bovine BLG existed in two genetic forms that differed slightly in their electrophoretic behavior on paper at pH 8.6. These forms are called β -lactoglobulin-A (BLG-A) and β -lactoglobulin-B (BLG-B).⁸ Although several other BLG genetic variants exist, A and B are predominant. Variant A differs in amino acid sequence from variant B at position 64 (AspA \rightarrow GlyB) and 118 (ValA \rightarrow AlaB). These differences result in distinct biophysical and biochemical properties of the variants, such as heat stability, self-association properties, and solubility.⁹ The

difference in the thermal behavior can be explained by the destabilization of the core of the B variant relative to the A variant due to the cavity formed by the loss of the two methyl groups due to the substitution of Val/Ala in BLG-A/BLG-B. BLG has also been implicated in allergy to bovine milk, and it has been shown that the immune response in mice is stronger for the B variant than for the A variant.^{9–11}

Platinum drugs have played a key role among the metal-based anticancer agents. The initial discovery in 1969 of the antitumor properties of cisplatin (diammine dichloroplatinum, cis-PtCl₂(NH₃)₂) by Rosenberg¹² was suddenly followed by clinical trials demonstrating its efficacy toward a variety of solid tumors.¹³ Presently, platinum(II) complexes including cisplatin and carboplatin are widely used in treating a number of human malignancies, such as ovarian, testicular, lung, urinary bladder, head, and neck cancers.^{14–16} However, cisplatin cause several types of dose-limiting toxicity, including nephrotoxicity (kidney damage), neurotoxicity (nervous system damage), and ototoxicity (hearing loss). Other side effects recorded include elevated blood pressure, diarrhea, severe nausea, and vomiting.^{13,14}

Because palladium chemistry is similar to that of platinum, it was speculated that Pd complexes might also exhibit antitumor activities with fewer side effects. Attempts have been made to synthesize Pd(II) complexes with such activities. Palladium complexes are expected to have less kidney toxicity than cisplatin, which results from binding of the sulphydryl groups of kidney tubule protein.^{15–17} Our group¹⁴ and others^{13,18,19} have

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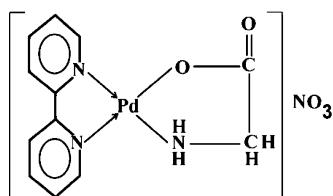


Figure 1. Molecular structure of the Pd(II) complex.

reported that Pd(II) complexes also have significant cytotoxicity against certain human tumor cell lines. Genova et al.²⁰ have shown that Pd(II) complexes exhibit a significant activity against acyclovir-resistant viruses R-100 (HSV 1) and PU (HSV 2) and negatively influence the expression of key structural HSV-1 proteins (VP 23, gH, and gG/gD), thus simultaneously suppressing virus entry, transactivation of virus genome, capsid assembly, and cell-to-cell spread of infectious HSV progeny. In a previous paper, we demonstrated the ability of BLG to transport Pd(II) complexes^{11,14} that were synthesized in our lab and determined the number of binding sites and the binding constant for these complexes on the carrier protein BLG. We then investigated the cytotoxic and apoptotic effects of these complexes, characterizing the antitumor activity. In the present study, we try to characterize biological evaluations of one of the these complexes, 2,2'-bipyridinglycinato Pd(II) nitrate, hereafter referred to as the Pd(II) complex, the molecular structure of which is shown in Figure 1. Whey carrier protein of β -lactoglobulin-B (BLG-B) and chronic myelogenous leukemia cell line K562 were selected as the targets.

Experimental Section

Materials. Bovine BLG-B was purchased from the Sigma. All other materials and reagents were of analytical grade. All solutions were made in double-distilled water. Since the Pd(II) complex does not dissolve in any buffers, NaCl solution (5 mM) was used as a solvent to prepare the Pd(II) complex solution. Concentrations of BLG-B were determined spectrophotometrically using a value of $17\,600\text{ M}^{-1}\text{ cm}^{-1}$ for the molecular absorption coefficient at 278 nm (ϵ_{278}).²¹

Preparation of 2,2'-Bipyridinelycinatopalladium(II) Chloride [Pd(bpy)(Gly)]Cl. This compound was prepared by the method described by Puthraya and Srivastava^{22,23} with some modifications: NaHCO₃ (84 mg, 1 mmol) and glycine (75 mg, 1 mmol) were dissolved in 10 mL of water and stirred for 20 min at 60 °C and cooled. This solution was added to a suspension of [Pd(bpy)Cl₂] (333 mg, 1 mmol) in 40 mL of methanol/water (2:1 v/v) mixture. The reaction mixture was stirred overnight at 50 °C under reflux. The resulting yellow solution was filtered to remove any insoluble material, and the filtrate was concentrated to 5 mL at 40 °C. This concentrated solution was cooled to 5 °C in a refrigerator to give pale yellow crystals. These crystals were filtered and recrystallized from water, washed with acetone, and finally dried in an oven at 40–50 °C. Yield was 312 mg (84%). Anal. Calcd for C₁₂H₁₂N₄O₅Pd (398): C, 36.18; H, 3.02; N, 14.07%. Found: C, 36.14; H, 3.00; N, 14.09%. Solid state IR spectroscopy of the above compound shows a characteristic band at 1660 cm⁻¹ assigned to coordinated ν(COO⁻) mode.²³ ¹H NMR (500 MHz, DMSO-*d*₆, δ in ppm; s, d, t, and sb stand for singlet, doublet, triplet, and singlet broad), 3.88 (s, 2H, H-a), 6.32 (sb, 2H, H-b), 8.32 (d, 2H, H-6,6'), 7.72 (t, 2H, H-5,5'), 8.59 (d, 2H, H-4,4'), and 8.36 (t, 2H, H-3,3') (Figure 1). Molar conductance of 10⁻³ M solution is 94.00 cm² Ω⁻¹ mol⁻¹, UV-vis maxima in water: 322 nm ($\epsilon = 0.79\text{ mol}^{-1}\text{ cm}^{-1} \times 10^{-4}$) and 293 nm ($\epsilon = 0.85$

are metal to bpy charge transfer and 310 nm ($\epsilon = 1.28$), 242 nm ($\epsilon = 1.58$), and 208 nm ($\epsilon = 3.13$) are $\pi \rightarrow \pi^*$ internal transitions of bpy moiety.

Fluorescence Measurements. Fluorescence intensity measurements were carried out using a Hitachi model MPF-4 spectrofluorimeter at an excitation wavelength of 290 nm, and the emission spectra of all samples were recorded in the range of 300–470 nm at 27, 37, 42, and 47 °C. All measurements were made using a 1-cm path length fluorescence cuvette. The tryptophan fluorescence spectra of the BLG-B (5 μM) were also measured in the presence of 3 up to 54 μM of the Pd(II) complex.

Circular Dichroism (CD) Measurements. CD spectra were recorded using an Aviv model 215 spectropolarimeter. Changes in the secondary and tertiary structures of BLG at different temperatures of 27, 37, 42, and 47 °C were monitored in both the far- and near-UV regions (200–260 and 260–320 nm, respectively) using 1-cm path length cells, at protein concentrations of 13.5 and 50 μM, respectively. The results were expressed in mean residue ellipticity [θ] (deg cm² dmol⁻¹) based on a mean amino acid residue weight of 114 (MRW). The mean residue ellipticity at a wavelength, λ , was obtained using the relation $[\theta]_\lambda = (100 \times \text{MRW} \times \theta_{\text{obs}}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength λ , c is the protein concentration in mg/mL, and l is the path length in cm. The CD software was used to predict the secondary structure of the protein according to the statistical method.^{24,25}

Cytotoxic Studies

Cell Culture. In RPMI medium cells were grown. This medium was supplemented with L-glutamine (2 mM), streptomycin and penicillin (5 μg/mL), and 10% heat-inactivated fetal calf serum, at 37 °C under a 5% CO₂/95% air atmosphere.

Cell Proliferation Assay. The above Pd(II) complex inhibits the growth of chronic myelogenous leukemia cell line K562. This growth inhibition was measured by means of MTT assay.¹⁴ The cleavage and conversion of the soluble yellowish MTT to the insoluble purple formazan by active mitochondrial dehydrogenase of living cells has been used to develop an assay system alternative to other assays for measurement of cell proliferation. Harvested cells were seeded into a 96-well plate (1 × 10⁴ cell/mL) with varying concentrations of the sterilized drugs (0–250 μM) and incubated for 24 and 48 h. Four hours to the end of incubations, 25 μL of MTT solution (5 mg/mL in PBS) was added to each well containing fresh and cultured medium. At the end, the insoluble formazan produced was dissolved in solution containing 10% SDS and 50% DMF (left for 2 h at 37 °C in dark conditions), and optical density (OD) was read against reagent blank with multiwell scanning spectrophotometer (ELISA reader, model Expert 96, Asys Hitchech, Austria) at a wavelength of 570 nm. Absorbance is a function of the 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%).

Apoptotic Assay. To ensure that significant under- or overestimation of cell death was not occurring, the percentages of apoptotic and necrotic cells were determined by analyzing phosphatidylserine externalization and membrane integrity by double staining with Annexin V and PI using flow cytometry as previously described^{26,27} with a commercially available staining kit. Positioning of quadrants on Annexin V/PI dot plots was performed, and living cells (Annexin V-/PI-), early apoptotic/primary apoptotic cells (Annexin V+/PI-), late apoptotic/secondary apoptotic cells (Annexin V+/PI+), and

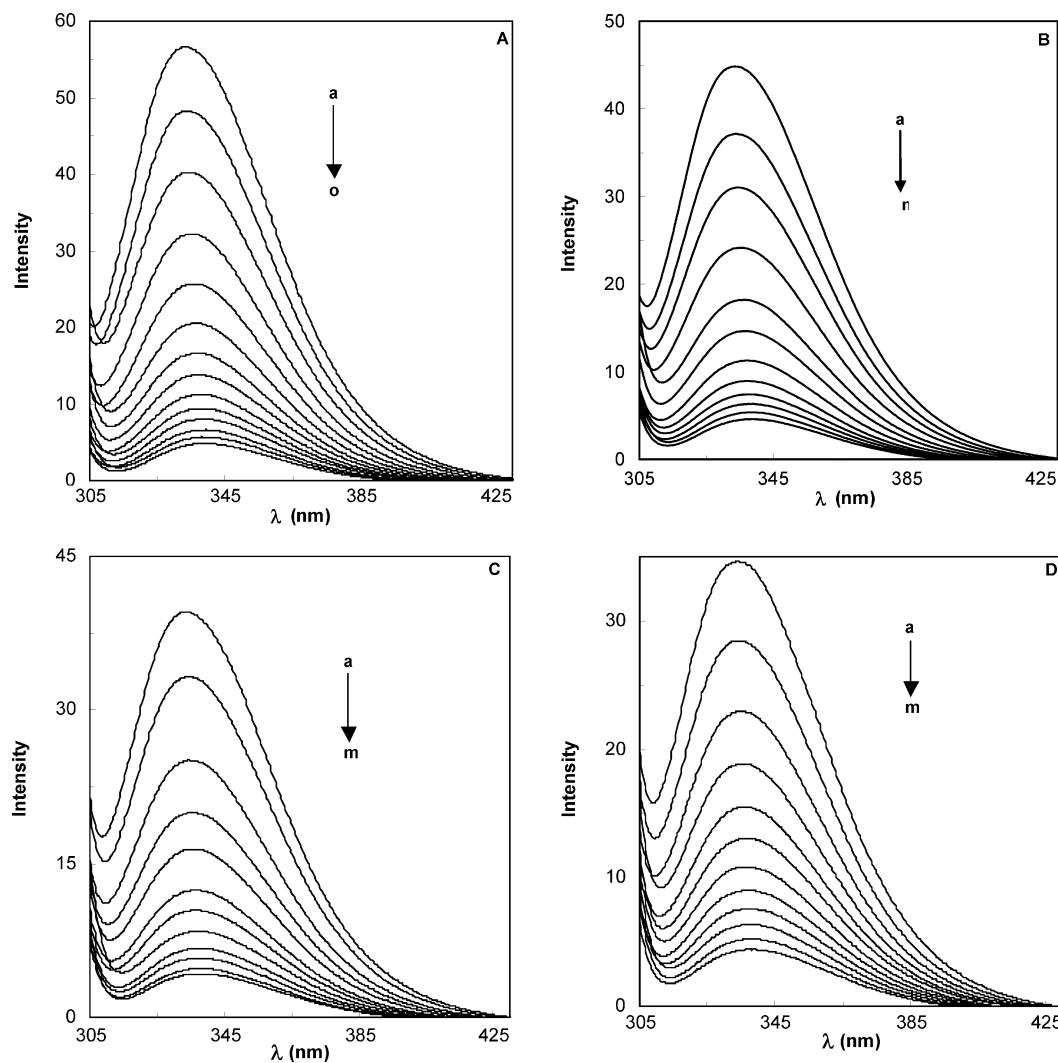


Figure 2. Fluorescence titration curve of BLG-B ($5 \mu\text{M}$) with different concentrations of Pd(II) complex (0 (a), 3 (b), 6 (c), 12 (d), 14.9 (e), 17.8 (f), 20.8 (g), 26.7 (h), 29.7 (i), 32.6 (k), 35.6 (l), 39.5 (m), 49 (n), and $54.5 \mu\text{M}$ (o)) at different temperatures of 27 (A), 37 (B), 42 (C), and 47 °C (D) in 5 mM sodium chloride solution.

necrotic cells (Annexin V-/PI+) were distinguished.²⁷ Therefore, the total apoptotic proportion included the percentage of cells with fluorescence Annexin V+/PI- and Annexin V+/PI+. Annexin V binding was performed using an Annexin V-FITC kit (IQ product, Netherlands) as described by the manufacturer. Cells were plated at a density of 10^6 cells/well into 24 well plates for 24 and 48 h. The cells were pretreated with various concentrations of the Pd(II) complex (Cc_{50} and 2Cc_{50}). After 24 and 48 h, the cells were harvested and washed with PBS and suspended in $100 \mu\text{L}$ of Annexin V binding buffer (containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Then, the cells were double stained with $10 \mu\text{L}$ FITC-labeled Annexin V and $10 \mu\text{L}$ PI solution (containing 50 $\mu\text{g}/\text{mL}$ in PBS). The samples were incubated for 20 min at room temperature and then analyzed by flow cytometry (Partec PAS, Germany).

Statistical Analysis. Results were analyzed for statistical significance using two tailed student's *t* tests. Changes were considered significant at $p < 0.05$.

Results and Discussion

Effects of the Pd(II) complex on the structure of the B variant of carrier protein, BLG-B, was investigated, using intrinsic fluorescence and CD spectroscopy.

Fluorescence Studies. Fluorescence spectroscopy is a useful technique to study the structure, dynamics, and binding properties of protein molecules in solution. The intrinsic fluorescence of tryptophanyl residues is a particularly sensitive method for this kind of study, as BLG has two tryptophanyl residues. From crystal structure studies,²⁸ Trp19 is at the base of the central hydrophobic calyx of the protein, while Trp 61 is part of an external loop. The intrinsic fluorescence of BLG is then almost exclusively attributed to Trp19, positioned in a more apolar environment than Trp61.²⁹ Parts A–D of Figure 2 show the intrinsic fluorescence of the BLG in the presence of different concentrations of Pd(II) complex at different temperatures of 27, 37, 42, and 47 °C. As shown in Figures 2A–D, the Pd(II) complex reduces the intrinsic fluorescence emission of BLG markedly at different concentrations and then quenches it, and red shifts (approximately 4–5 nm) are observed in a maximum emission wavelength (λ_{max}), which may be related to the loss of compact structure in the hydrophobic regions where Trp residues are located.^{30,31} Figure 3 shows the intrinsic fluorescence at 335 nm of BLG-B at different temperatures and concentrations of the Pd(II). It should be noted that the Pd(II) complex had negligible fluorescence intensities even at the highest concentration. Reports have shown that the fluorescence intensity of a compound can be decreased by a variety of molecular interactions, including excited-state reactions, molecular rearrange-

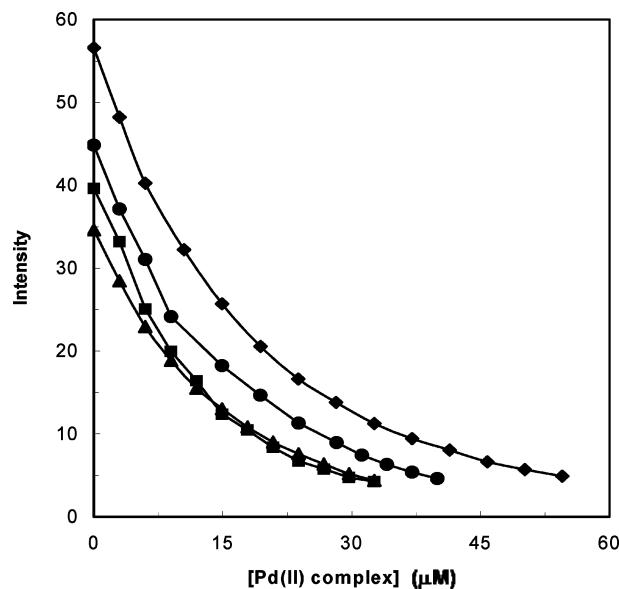


Figure 3. Fluorescence titration curve of BLG-B ($5 \mu\text{M}$) with Pd(II) complex in 5 mM NaCl solution, pH 7, at 27°C (◆), 37°C (●), 42°C (■), and 47°C (▲).

ments, energy transfer, ground-state complex formation, and collision. The role of this decrease in intensity, called fluorescence quenching,^{23–26} can be studied experimentally by determining quenching rate parameters using Stern–Volmer plots.³² The intrinsic fluorescence of BLG-B is strongly quenched by the addition of the Pd(II) complex. Furthermore, the fluorescence emission of the protein is fully quenched at lower concentrations of the Pd(II) complex when the temperature is increased (Figure 3). In order to speculate on the fluorescence quenching mechanism, the fluorescence quenching data for BLG-B was first analyzed using the classic Stern–Volmer equation

$$\frac{F_0}{F} = 1 + K_{\text{SV}}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of the BLG-B variant in the absence and presence of the Pd(II) complex, respectively. K_{SV} is the Stern–Volmer dynamic quenching constant, and $[Q]$ is the total concentration of the quencher (in this case, the Pd(II) complex). Using eq 1, the fluorescence quenching data for BLG-B show positive deviation for the plots of F_0/F versus $[Q]$ (see Figure 4), where the binding of the Pd(II) complex to BLG-B results in a quenching of the fluorophore, probably initiated by complex formation rather than dynamic collision.

For static quenching, the following equation was employed to calculate the binding constant and the number of binding sites³²

$$\frac{F_0}{F} = K_A \frac{[Q]F_0}{F_0 - F} - nK_A[P]_t \quad (2)$$

where F_0 and F are the fluorescence intensities of BLG in the absence and presence of the quencher (Pd(II) complex), respectively, K_A is the apparent association constant (binding constant) for the equilibrium formation of the BLG–Pd(II) complex, n is the number of binding sites, and $[Q]$ and $[P]_t$ are the total concentrations of quencher and the protein (BLG-B), respectively. Thus, a plot of F_0/F versus $[Q] \times F_0/(F_0 - F)$

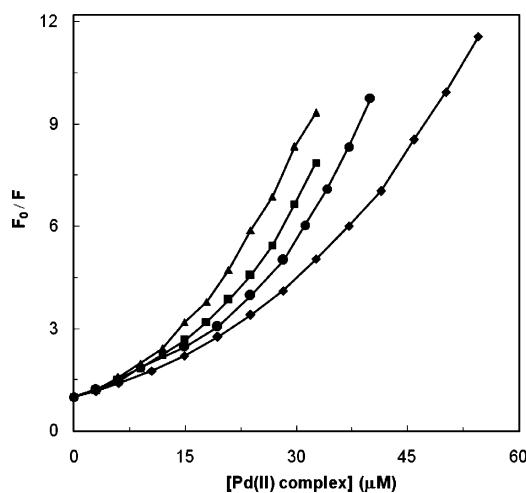


Figure 4. Stern–Volmer curve for quenching of Pd(II) complex to BLG-B in 5 mM NaCl solution at 27°C (◆), 37°C (●), 42°C (■), and 47°C (▲).

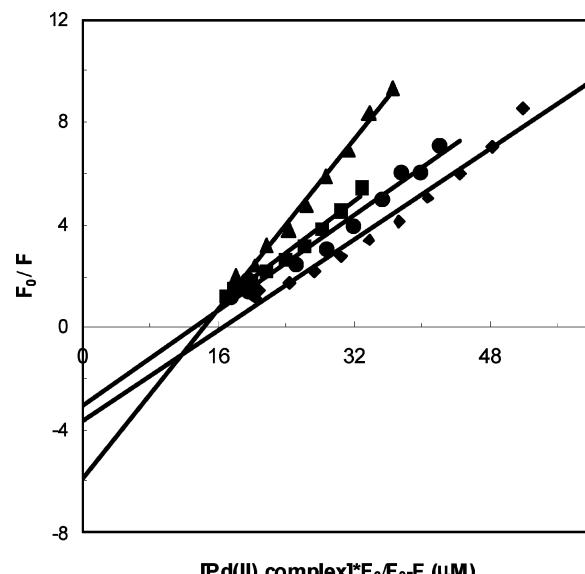


Figure 5. Best linear plot of F_0/F versus $[Q] \times (F_0/F_0 - F)$ according to the eq 2. Values of K_A and n of binding of Pd(II) complex to BLG-B can be obtained from the slope and the vertical intercepts, respectively, at 27°C (◆), 37°C (●), 42°C (■), and 47°C (▲).

TABLE 1: Various Parameters of BLG-B upon Interaction with Pd(II) Complex at Different Temperatures of 27, 37, 42, and 47 °C

temperature (° C)	n^a	K_A ($\times 10^6 \text{ M}^{-1}$) ^b	ΔG° (kJ/mol) ^c	ΔH° (kJ/mol) ^d	ΔS° (J/mol·K) ^e
27	3.3	0.22	-30.7		120.6
37	2.6	0.23	-31.9		120.6
42	2.8	0.41	-33.9	+5.51	-
47	2.5	0.25	-33.1		120.6

^a Number of binding sites. ^b Apparent association constant.

^c Values of the Gibbs free energy (ΔG°).

^d Values of the enthalpy.

^e Values of entropy of binding.

can be used to determine K_A as well as n . The binding data (K_A and n) for BLG-B represented in Figure 5 are shown in Table 1. It was found that, by increasing the temperature within the experimental range, the values of K_A increased, except at 42°C . An increase in K_A values indicates an increase in the affinity of the protein for the Pd(II) complex. As shown in Table 1 and Figure 4, increasing the temperature from 27 up to 47°C

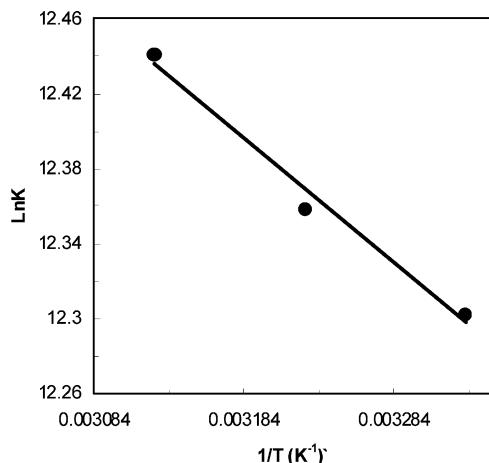


Figure 6. Van't Hoff plot for the binding of Pd(II) complex with BLG-B.

induced higher affinity of protein for complex. But in the case of 42 °C, which is the same as fever temperature, the K_A value suddenly increased and was repeatable after three times test. The reason for this fluctuation might be related to structural and functional changes of protein at this temperature, and then

the complex might be transferred by the protein with higher affinity relative room, physiologic, and upper fever temperatures. Therefore, it might be concluded that temperature has an important role in binding and transferring affinity of carrier protein of BLG. The association constants calculated for the different Pd(II) complexes—protein have shown strong ligand–protein complexes with binding constants ranging from 10^6 to $10^8 M^{-1}$. However, lower binding constants (10^4 – $10^5 M^{-1}$) were recently reported for several other drug–protein complexes using fluorescence spectroscopic methods. There are several reports that like as our results represent one, two, or three molecules of drug or ligand bound to carrier protein via forming strong ligand–protein complexes with binding constants ranging from 10^6 to $10^8 M^{-1}$.^{33,34}

Determination of Acting Force between Pd(II) Complex and BLG. Considering the dependence of the binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the BLG–Pd(II) complex.^{32,33} Thus, in order to further characterize the forces acting between the Pd(II) complex and BLG, the dependence of thermodynamic parameters on temperature was analyzed. The forces acting between a small molecule and macromolecule include hydrogen bond, van der Waals force, electrostatic force, and hydrophobic interaction, among others.^{32,35} The thermodynamic binding

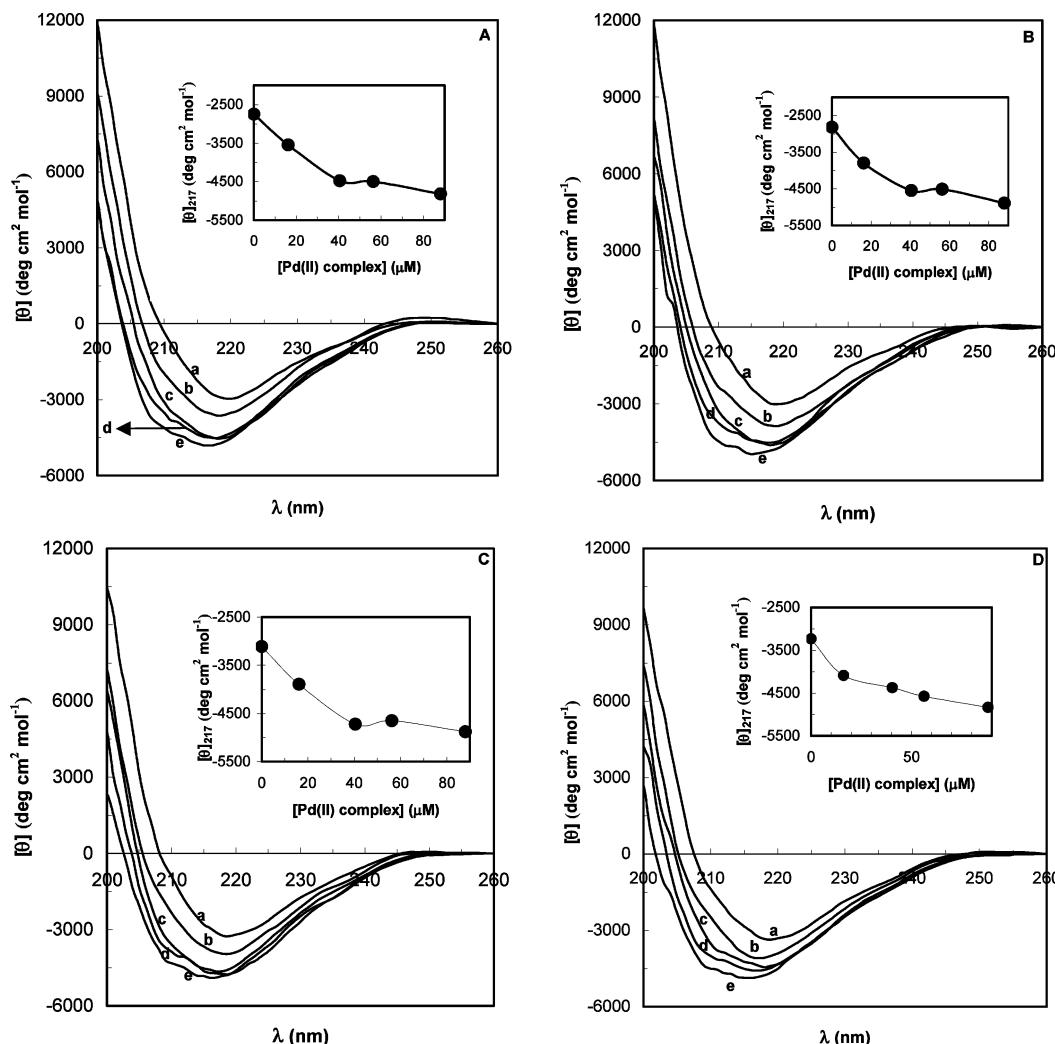


Figure 7. Far-UV circular dichorism spectra of 13.5 μM BLG-B measured in the absence (a) and presence of different concentrations of Pd(II) complex: 15 (b), 40 (c), 56 (d), and 88 μM (e) at different temperatures of 27 (A), 37 (B), 42 (C), and 47 °C (D) in 5 mM sodium chloride solution. The inset shows the changes of the ellipticity at 217 nm upon titration with different concentrations of Pd(II) complex.

parameters of enthalpy change (ΔH°) and entropy change (ΔS°) were determined using the van't Hoff equation:

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

The $\ln K_A$ versus $1/T$ plots (Figure 6) enable the determination of ΔH° for the binding process. Using the results from eq 3, the values of Gibbs free energy change (ΔG°) and entropy (ΔS°) are calculated:

$$\Delta G^\circ = -RT \ln K_A \quad (4)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

where R is the gas constant, the values of ΔH° , ΔS° , and ΔG° are given in Table 1, and T is the temperature at which the data was collected: 27, 37, 42, and 47 °C.

As shown in Table 1, the fact that $\Delta G^\circ < 0$ indicates that the binding of the Pd(II) complex to the BLG-B is a spontaneous intermolecular reaction. It can also be seen in Table 1 that the formations of the Pd(II)-BLG complexes are endothermic reactions accompanied by positive enthalpy (ΔH°) and positive entropy (ΔS°), which indicates that the binding processes are mainly entropy driven and the enthalpy is unfavorable for them. Therefore, the thermodynamic parameters for the interaction of Pd(II) complex and BLG can be explained on the basis of hydrophobic forces.^{24,27,31} However, in this study, ΔG° values were mainly due to the contribution of ΔS° but not ΔH° . Therefore, it can be concluded that hydrophobic interaction might play a major role in the interactions of the Pd(II) complex with BLG-B.

CD Studies. CD has proven to be an ideal technique for monitoring conformational changes in proteins, which can occur as a result of changes in experimental parameters such as pH, temperature, and ligand binding, among others.³⁶ While the secondary structure of proteins are characterized using the peptide bond absorption in the far-UV CD spectra,³⁷ the α -helix content of proteins is typically determined with the mean residue

TABLE 2: Changes in Secondary Structure of BLG-B upon Interaction with Different Concentrations of Pd(II) Complex at Different Temperatures

Pd(II) concentration (μ M) at different temperatures (°C)	% α -helix	% β -sheet	% random coil
0	27	15.3	42.3
	37	15.2	42.5
	42	15.5	42.4
	47	15.4	42.6
15	27	16.2	43.3
	37	16.1	42.5
	42	16.1	43.6
	47	16.3	42.5
40	27	16.2	44.6
	37	16.5	43.5
	42	16.8	44.3
	47	16.1	43.9
56	27	16.3	44.7
	37	16.6	42.8
	42	16.5	45.0
	47	16.5	44.1
88	27	16.2	45.2
	37	16.4	43.1
	42	15.7	45.8
	47	15.5	43.9

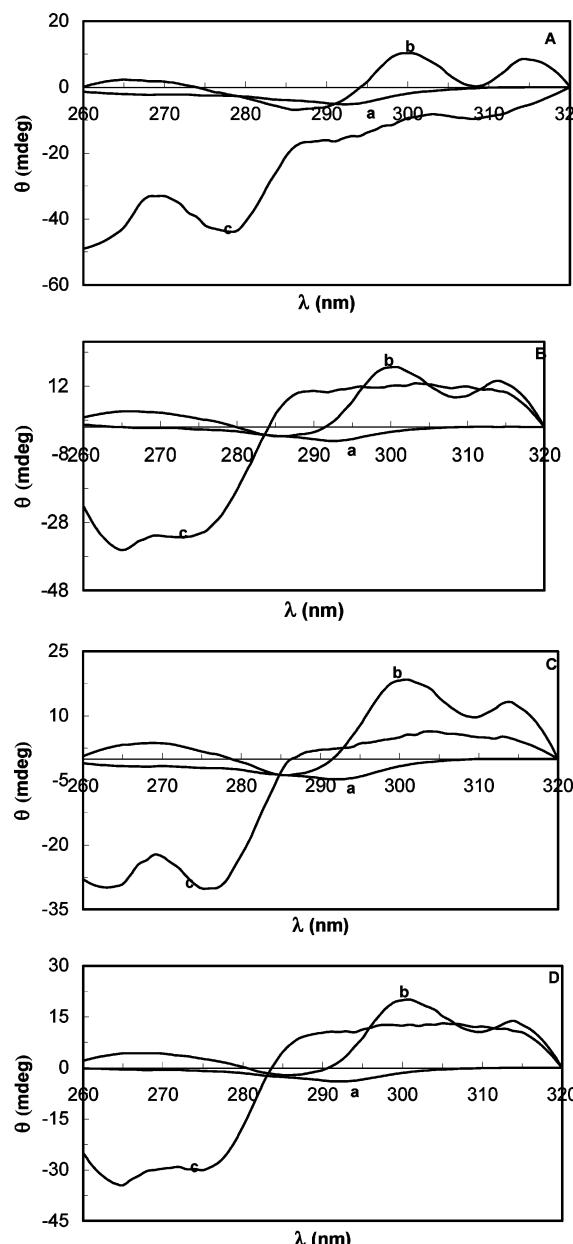


Figure 8. Near-UV circular dichroism spectra of $50 \mu\text{M}$ BLG-B measured in the absence (a) and presence of different concentrations of Pd(II) complex: 171 (b) and $540 \mu\text{M}$ (c) at different temperatures of 27 (A), 37 (B), 42 (C), and 47 °C (D) in 5 mM sodium chloride solution.

ellipticities at 222 nm ($[\theta]_{222}$) and 208 nm ($[\theta]_{208}$) or the ratio of $[\theta]_{208}/[\theta]_{222}$. The BLG CD spectrum is typical of a protein that is composed of antiparallel β -structure and shows a minimum at 217 nm .³⁸

The far-UV CD spectra of BLG-B in the absence and presence of the Pd(II) complex at different temperatures are shown in Figure 7. The insets show the changes in $[\theta]_{217}$ for each protein upon titration with the Pd(II) complex. Changes in secondary structure of BLG-B upon interaction with different concentrations of Pd(II) complex at different temperatures are shown in Table 2. From the CD data (Figure 7 and Table 2), it can be seen that, as the concentration of the Pd(II) complex increases, the values of $[\theta]_{217}$ and $[\theta]_{222}$ become more negative, up to $40.5 \mu\text{M}$, after which θ values plateau. It can, therefore, be concluded that certain concentrations of the Pd(II) complex can significantly change and stabilize the secondary structure

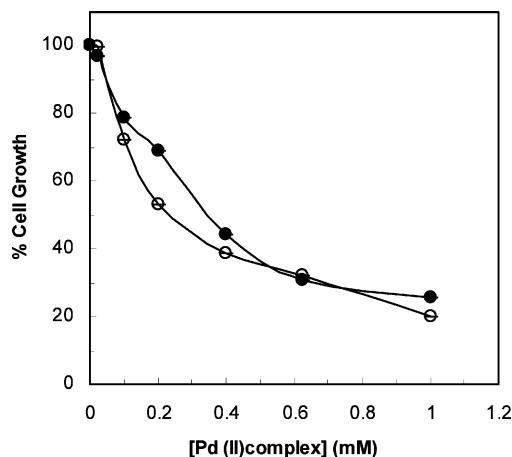


Figure 9. Growth suppression activity of the Pd(II) complex on K562 cell line was assessed using MTT assay as described in experimental section. The tumor cells were incubated with varying concentrations of the Pd(II) complex ranging from 0 to 1 mM for 24 (●) and 48 h (○). The values are the mean of three independent experiments.

of BLG-B, which is achieved by increasing the content of the regular secondary structure of the protein.

The near-UV CD spectra of proteins issue from the environment of each aromatic amino acid side chain in addition to any influence from disulfide bonds or nonprotein cofactors that might absorb in this spectral region, all of which provide information about the tertiary structure of the protein.³⁶ The near-UV CD spectrum of the BLG-B (Figure 8) has arrangements of negative peaks between 250 and 300 nm resulting from two tryptophans (Trp19 and 61), four tyrosines (Tyr 20, 42, 99, and 102), and four phenylalanines (Phe 82, 105, 136, and 151). All of these may contribute to the CD spectrum; however, the chief determinants of the near-UV CD curve are often Trp residues. While the less intense signals of Tyr and Phe generally do not absorb above 270 and 290 nm, respectively, the stronger Trp

signals occur between 250 and 300 nm.^{34–36} Thus, the negative bands at 293 and 285 nm can be attributed to asymmetrically perturbed tryptophans, whereas peaks below 280 nm can be assigned to the chiral environment of Phe and Tyr residues.³⁸

As it shown in Figures 8, two negative bands at 285, 293, and below 280 nm significantly changed with increasing Pd(II) complex at different temperatures, suggesting that the interaction of Pd(II) complex with BLG-B disrupts the anisotropic environment of Trp and Tyr residues³¹ that has a good agreement with fluorescence data. These figures further show that the addition of the Pd(II) complex significantly changes the near-UV CD spectrum of the native BLG, thus indicating a change in the tertiary structure of the protein.

Cytotoxicity Measurements of the Pd(II) Complex. The *in vitro* antitumor property of the synthesized Pd(II) complex was studied by testing them on human tumor cell line K562. In this study, various concentrations of Pd(II) complex ranging from 0 to 1 mM were used to culture the tumor cell lines for 24 and 48 h (Figure 9). The 50% cytotoxic concentration (C_{50}) of complex was calculated to be 0.345 and 0.215 mM for 24 and 48 h, respectively. As shown in Figure 9, the cell growing after 24 and 48 h was significantly reduced by various concentrations of the complex. Also treatment of the cells with Pd(II) complex of more than 100 μ M led to a marked decrease of cell proliferation as determined using MTT assay. Based on Figure 9, it is also clear that the Pd(II) complex produced a dose-response and time incubation sensitive suppression on growing of K562 leukemia cell lines. It can be noticed that increasing of incubation time of Pd(II) complex has a great influence on the growth suppression activity of this complex on K562 cells. Several reports have represented that pyrazole-containing complexes have been reported to possess antitumor activity which is comparable with that of cisplatin, and then Miernicka³⁹ et al. have substituted pyrazoles as analytical reagents in the complexation of transition metal ions such as Pd, Pt, and Cu. Their cytotoxicity assay on K562 cell lines

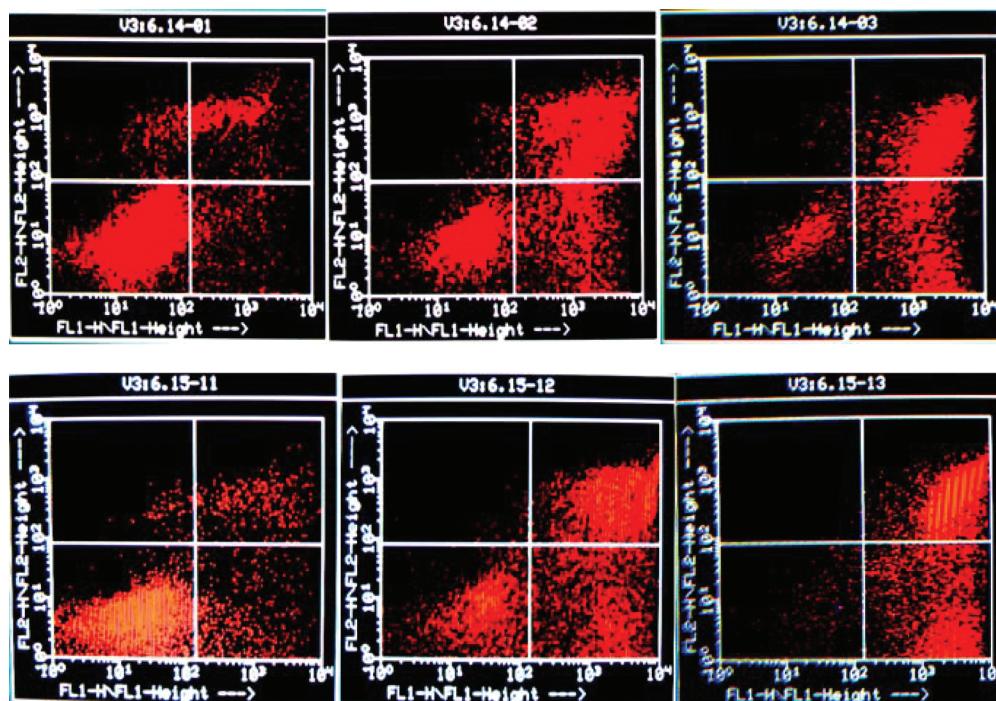


Figure 10. Dot plots of PI versus Annexin V staining followed by flow cytometry analyses for K562 at density of 10^6 cell/mL, based on Pd(II) complex-induced externalization of phosphatidylserine. Figures from left to right represent controls of K562 cells without treatment (left) and K562 cells treated with C_{50} and $2C_{50}$ concentration of Pd(II) complex after 24 (up) and 48 h (down).

TABLE 3: Percentage of Living Cells, Early Apoptotic/Primary Apoptotic Cells, Late Apoptotic/Secondary Apoptotic Cells, and Necrotic Cells in the Absence and Presence of Different Concentrations of Pd(II) Complex after 24 and 48 h Incubation Time

cells	UL ^a 24 h	UR ^b 24 h	LL ^c 24 h	LR ^d 24 h	UL 48	UR 48 h	LL 48 h	LR 48 h
control	3.17	7.07	83.12	6.65	1.332	5.21	83.45	10.03
Pd-complex (Cc ₅₀)	1.16	37.95	33.77	27.13	0.59	35.05	23.81	40.56
Pd-complex (2Cc ₅₀)	1.1	41.61	15.52	41.77	0.1	45.49	2.50	51.91

^a Percentage of necrotic cells (Annexin V-/PI+). ^b Percentage of late apoptotic/secondary apoptotic cells (Annexin V+/PI+). ^c Percentage of living cells (Annexin V-/PI-). ^d Percentage of early apoptotic/primary apoptotic cells (Annexin V+/PI-).

showed lower cytotoxicity than cisplatin, and the Pt(II) and Cu(II) complexes were found to be more efficient in the induction of leukemia cell death than the Pd(II) complex (with Cc₅₀ = 110 μM). It can be concluded that our synthesized complex has lower cytotoxicity against leukemia cell line relative to pyrazole-containing Pd complexes.

Apoptotic Measurements. Since many of the current treatments have problems with toxicity and drug resistance, there is a strong demand for the discovery and development of effective new cancer therapies. Identification of compounds that activate and promote apoptosis is one of the attractive approaches for the discovery and development of potential anticancer agents.^{40–43} Therefore, modern drug design is often based on studies detecting the abilities of investigated complexes to induce apoptosis as the preferred mode of cell death.

Then, we next sought to determine whether the decrease in viability of K562 cells is due to apoptosis or other modes of cell death. In the earlier stages of apoptosis, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V, Ca²⁺-dependent phospholipid-binding protein with a high affinity for PS,^{39,40} was used in order to examine whether treatment of K562 cells with Pd(II) complex induces externalization of PS. K562 cells were treated at different times (24 and 48 h) with (1 Cc₅₀ and 2 Cc₅₀) concentrations of Pd(II) complex. Representative results of flow cytometry analysis for treatment of K562 with Pd(II) complex for 24 and 48 h are shown in Figure 10. These data show that incubation of K562 cells with Pd(II) complex can decrease the viable population while the percentages of apoptotic cells were significantly increased by treatment with Pd(II) complex while the percentage of necrotic cells did not show significant alterations. Also, prolonged treatment of the cells with the same concentration of Pd(II) complex from 24 to 48 h apparently resulted in an increase of the apoptotic cell percentage from 65.08 to 83.38 for Cc₅₀ and 75.61 to 97.4 for 2Cc₅₀ Pd(II) concentration (Figure 10 and Table 3).

Above results show that our newly designed Pd(II) complex induces apoptosis of K562 cells in a concentration and time dependent manner.

Conclusions

This study shows that the Pd(II) complex binds to BLG-B and quenches the fluorescence spectra of the protein at different temperatures. The Stern–Volmer plots of the protein in the presence of this complex at different temperatures are nonlinear, showing positive deviation. Binding studies indicate an increase in the affinity of the protein for the Pd(II) complex as temperatures increase. Far- and near-UV CD studies show that an appropriate concentration of Pd(II) complex induces conformational changes in the secondary and tertiary structures of BLG-B at different temperatures.

The cytotoxicity and biochemical changes on human leukemic K562 cells induced by this new Pd(II) complex have not been

reported previously and could therefore be recorded as a novel biological activity of the newly designed anticancer compound. Additionally, these results suggest that our newly synthesized Pd(II) complex is a promising antiproliferative agent and should execute its biological effects by inducing apoptosis. Understanding the mechanisms by which antiproliferative drugs induce cell death by apoptosis is part of a strategy to treat human tumors; therefore, studies concerning the mechanisms of the apoptotic effects of this metal complex on K562 cancer cells merit to be further clarified by *in vitro* and *in vivo* studies.

The biological significance of this work may be evident since BLG serves as a carrier molecule for several antitumor compounds. Therefore, the interaction of the Pd(II) complex (with antitumor activity) can provide useful information to better design metal anticancer complexes with fewer side effects.

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