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Effects of Vitamin K3 on Cytotoxicity and DNA-binding Properties of a Palladium(II) Complex as a Potential Antitumor Agent

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ABSTRACT

In many diseases such as cancer, simultaneous use of two or more pharmacologically active agents will be more effective and have fewer side effects. In this study a new palladium(II) complex with formula of [Pd(phen)(py-dtc)]NO_3 (where is phen 1,10-phenanthroline and py-dtc is n-propyldithiocarbamate), was synthesized. The cytotoxic activity of this complex was tested against leukemia K562 cell lines. The cytotoxic concentration (Cc50) value of above Pd(II) complex was 53.06 µM. In the presence of vitamin K3, Cc50 value dramatically decreased to 32.95 µM and encouraged us to study DNA interaction of Pd(II) complex in the absence and presence of vitamin K3. Interaction of above Pd(II) complex in two modes (in the absence and presence of vitamin K3) with calf thymus DNA was evaluated using spectroscopic methods. UV-Vis results showed that in the presence of VK3, Pd(II) complex unexpectedly denatures CT-DNA at very low concentration. Fluorescence results showed that quenching of the intrinsic fluorescence of EBr-DNA system by Pd(II) complex is static quenching mechanism and type of mechanism does not change in the presence of vitamin. Also, in the absence and presence of vitamin, the mode of intercalation might play a major role in the interactions of Pd(II) complex with DNA. Several binding and thermodynamic parameters are also presented. We hope that these results provide a basis for additional studies and clinical use of combined anticancer drugs to be useful.

Keywords: Anticancer; Pd(II) complex; Vitamin K3; Cytotoxicity; DNA binding

INTRODUCTION

Vitamin K as a group of fat-soluble compound is essential nutrient for blood clotting. Vitamin K includes two natural vitamers (vitamin K1 and vitamin K2) and a synthetic form (vitamin K3, VK3) that the synthetic form (vitamin K3) has shown toxicity. *In vitro*, Vitamin K3 is effective against a wide variety of tumor cells at concentrations that are clinically achievable Cc_{50} (50% cytotoxic concentration) values usually ranging from 10 to 50 µM [1,2]. This synthetic vitamin in combination with vitamin C, achieved cytotoxic dosages 5-10 times lower than when singly administered [3]. Both *in vivo* and *in vitro* studies showed a synergistic effect when VK3 was combined with conventional chemotherapeutic agents. Combining VK3 (5×10^{-7} to 5×10^{-6} M) and 5-fluorouracil (5-FU) significantly enhanced the action against hematoma cells [4]. Furthermore, VK3 was found synergistic with 5-FU, bleomycin, cisplatin and decarbonize in human oral epidermis carcinoma cell culture.

On the other hand, to reduce the toxicity of cisplatin and other platinum(II)-based drugs, sulphur, containing compounds (especially thiols and dithiocarbamates), was administered as antidotes [4-6]. This is perhaps due to the strong binding of platinum with dithiocarbamate, which prevents or at least limits the reaction with other sulfur-containing renal proteins [7].

In the continuation of our studies for the synthesis of effective anticancer drugs with fewer side effects, in this research we synthesized a palladium(II) square planar

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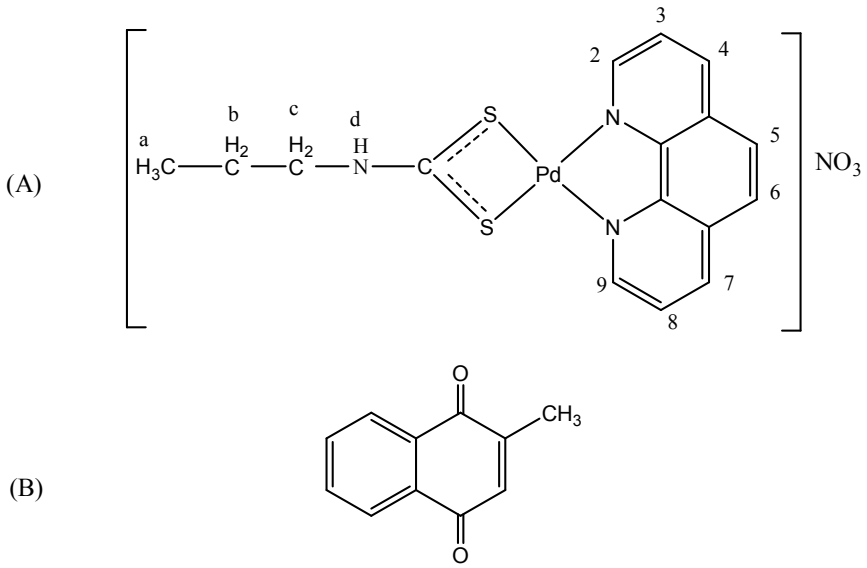

Fig. 1. Proposed structures of $[Pd(phen)(py\text{-}dtc)]NO_3$ and structures of vitamin K3.

complex containing dithiocarbamate moiety (see Fig. 1). Interestingly, the cytotoxic effects of this Pd(II) complex are significantly increased when it was combined with VK3. Thus, we used ultraviolet-visible (UV-Vis) absorption spectroscopy, fluorescence spectroscopy, and gel filtration techniques to explore the interaction mode between Pd(II) complex and Calf Thymus DNA (CT-DNA) in the presence and absence of VK3. In these interaction studies, several bindings and thermodynamic parameters have been described which may show the amount of changes in drug affinity in the presence of VK3.

EXPERIMENTAL SECTION

Chemicals

1,10-Phenanthroline, CS_2, SephadexG-25 and Tris-HCl (tris(hydroxymethyl) amino methane hydrochloride) were obtained from Merck (Germany). Calf thymus DNA, vitamin K3, n-propylamine, $AgNO_3$ and Ethidium bromide (EBr) were purchased from Sigma Chemical Co. (USA). Palladium(II) chloride anhydrous was bought from Fluka (Switzerland). $[Pd(phen)Cl_2]$ and n-propyldithiocarbamate sodium salt were prepared by the procedures described in the literature [8,9]. The cell line for the cytotoxicity studies was obtained from the Cell Bank of Pasteur Institute in Tehran (IRAN).

Concentration of CT-DNA in Tris-HCl buffer (20 mM) was determined spectrophotometrically at 260 nm using molar extinction coefficient ε_{260} = 6600 cm^{-1} M^{-1}. The ratio of the absorbance at 260 nm to that at 280 nm, A_{260}/A_{280} > 1.8, indicated that DNA was sufficiently pure and free from protein [10].

Instrumentation

Infrared spectra of the metal complex were recorded on a $J_{AS\cdot CO}$-460 plus FT-IR spectrophotometer in the range of 4000-400 cm^{-1} in KBr pellets. ^1H NMR spectra were recorded on a Brucker DRX-500 Avance spectrometer at 500 MHz in DMSO-d_6 using tetramethylsilane as an internal reference. Conductivity measurements of the above palladium complex were carried out on a Systronics conductivity bridge 305, using a conductivity cell of cell constant 1.0. UV-Vis spectra were recorded on a $J_{AS\cdot CO}$ UV-Vis-7850 recording spectrophotometer. Fluorescence spectra were carried out using a Varian spectrofluorimeter model Cary Eclips. All of the spectroscopic work was carried out at pH 7.4, maintained using a Tris-HCl buffer.

Preparation of $[Pd(phen)(py\text{-}dtc)]NO_3$

The preparation of the complex was carried out using the following procedure: Initially, $[Pd(phen)(OH_2)_2](NO_3)_2$ complex was prepared following the previous literature

procedure [11] to the clear yellow filtrate containing the above aqua complex (1.5 mmol), an aqua solution of sodium n-propyldithiocarbamate (1.5 mmol) was added. After 2 h, solution was filtered and the clear yellowish filtrate was evaporated at 35-40 °C. A yellow precipitate was obtained and recrystallized in dichloromethane/ methanol (1:1) solvent mixture and then dried in vacuum. Yield: 0.449 g (62%) and decomposed at 273 °C. Anal. Calcd. for $C_{16}H_{16}N_4O_3S_2Pd$ (482.4): C, 39.81; H, 3.32; N, 11.61%. Found: C, 39.66; H, 3.28; N, 11.53%. Solid state FT-IR spectroscopy of the above complex showed three distinguishing stretching bands at $1535_{\nu(N\text{-}CSS)}$, $87_{\nu(CSS)as}$ and $702_{\nu(CSS)s}$ cm^{-1} [12,13]. The uncoordinated NO_3^- ion show a sharp band at 1382 cm^{-1} [14]. ^1H NMR (500 MHz, DMSO-d_6, ppm, s = singlet, t = triplet, m = multiple): 0.93 (t, 3H, H-a), 1.61 (m, 2H, H-b), 3.32 (m, 2H, H-c), 11.54 (s, 1H, H-d), 7.84-8.75 (8H, 1,10-phenanthroline protons).Four bands were observed in electronic spectra: 216 (logε = 3.22), 244 (logε = 3.38), 272 (logε = 3.31) and 353 nm (logε = 2.24) which assigned to intraligand $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions of phenanthroline ligand as well as CSS^- group [15]. Molar conductance measurement for the complex is 159 Ω^{-1} mol^{-1} cm^2 indicating 1:1 electrolyte [16] (see Fig. 1).

Cytotoxicity Assay

A simple colorimetric assay using MTT (a yellow tetrazole that is reduced to purple formazan in living cells) was used to investigate the Cell proliferation. The MTT assay is dependent on the cleavage and conversion of the soluble yellowish MTT to the insoluble purple formazan by active mitochondrial dehydrogenase of living cells. Then, the insoluble purple formazan product is dissolved in solution containing 10% SDS and diluted HCl. Absorbance of this colored solution at 540 nm is a function of concentration of converted dye. The absorption maximum is dependent on the solvent employed. The leukemia K562 cell lines were maintained in an RPMI medium supplemented with 2 mM L-glutamine, streptomycin, and penicillin (5 μg ml^{-1}), at 37 °C under a 5% CO_2/95% air atmosphere. Harvested cells were seeded into 96-well plates (2×10^4 cell/ml) with varying concentrations of the complex (0-250 μM) in the absence and presence (with ratio of 1:1) of VK3 and incubated for 24 h. Four hours to the end of the incubations, 25 μl of MTT solution (5 mg ml^{-1} in PBS) was added to each well containing fresh cultured media [11]. The insoluble produced formazan was then dissolved in solution containing 10% SDS and 50% DMF (under dark condition for 2 h at 37 °C), and optical density (OD) was read against reagent blank with a multiwall scanning spectrophotometer (ELISA reader, Model Expert 96, Asys Hitchech, Austria). In this experiment, the clear stock solution (2 mM, in deionized water) was sterilized by filtering through sterilizing membrane (0.1 nm) and then varying concentrations of the sterilized complex (0-250 μM) were added to harvested cells.

DNA Binding Experiments

The DNA binding experiments of Pd(II) complex were performed in two modes: (i) in the absence of VK3 (ii) in the presence of VK3 (with ratio of 1:1). The stock solution of Pd(II) complex and VK3 (50 mM) were made in Tris-HCl buffer. To ensure of any reaction between the VK3 and Pd(II) complex, we have recorded the Pd(II) absorption changes with increasing different concentrations of VK3. After 24 h, no significant change was observed in the absorption spectra of Pd(II) complex which indicates that VK3 has no interaction with Pd(II) complex.

Electronic spectral studies. In DNA-denaturation experiment, the concentration of metal complex at midpoint of transition, $[L]_{1/2}$, in the absence and presence of VK3 was determined. The binding constant (K) and some of binding parameters such as Hill coefficient (n) and number of binding sites per 1000 nucleotides of DNA (g) for the interaction of Pd(II) complex and (Pd(II) complex + VK3) system with CT-DNA was obtained from absorption titration data by the procedures described in the literature [17].

Fluorescent spectral studies. The competitive experiments were conducted by adding small aliquots of Pd(II) complex and Pd(II) complex + VK3 stock solution to DNA-EBr (ethidium bromide) complex solution. The change of fluorescence intensity was recorded. The excitation wavelength was 471 nm and the emission spectra were observed between 540 and 700 nm. In addition, Scatchard analysis was conducted. In this experiment, the Pd(II) complex was incubated with CT-DNA in the presence and absence of VK3 at room temperature for 1 h. The appropriate amount of EBr was added before an

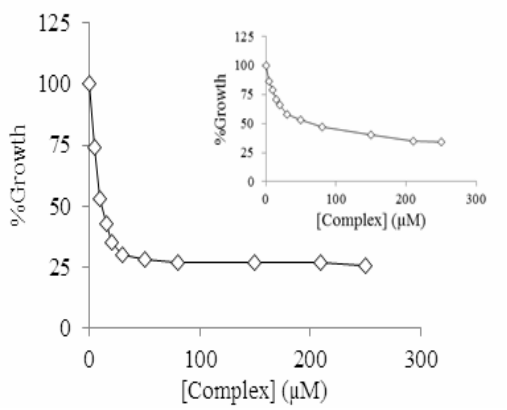

Fig. 2. The growth suppression activity of the [Pd(phen)(py-dtc)]NO_3 complex on K562 cell line (Insert for [Pd(phen)(py-dtc)]NO_3 in the presence of vitamin K3).

additional incubation for 2 h and then processed for final fluorescence spectral measurement.

Gel filtration. The Pd(II) complex and mixture of Pd(II) complex + VK3 were incubated with calf thymus DNA for 1 h at 27 °C in Tris-HCl buffer (pH 7.4) separately, and then passed through a Sephadex G-25 column equilibrated with the same buffer. The elusion of the column fraction of 2.0 ml was monitored at 303 nm for Pd(II) complex, 260 nm for DNA and 441 nm for VK3. Gel chromatograms are obtained by plotting the absorbance readings at the three wavelengths versus column fractions in the same plot.

RESULTS AND DISCUSSION

Cytotoxic Activity

The effect of Pd(II) complex on leukemia K562 cell lines in the absence and presence of VK3 were evaluated by MTT assay. As shown in Fig. 2, the number of growing cells was significantly reduced after 24 hours in the presence of various concentrations of the alone palladium complex. These results suggest that the Pd(II) complex may be potential antitumor agent. The Cc_{50} value of [Pd(phen)(py-dtc)]NO_3 obtained 53.06 µM and when it was combined with VK3 decreased to 32.95 µM. Also, the Cc_{50} value of cisplatin under the same experimental conditions was determined to be 154 µM which is much higher than that of prepared complex. The Cc_{50} value of [Pd(phen)(py-dtc)]NO_3 is comparable with those of other analogous Pt(II) and Pd(II) dithiocarbamate complexes reported earlier [18-20]. Due to the significant reduction in values of Cc_{50} of Pd(II) complex against leukemia K562 cells in the presence of vitamin K3, we studied the effect of VK3 on interaction of the Pd(II) complex with CT-DNA, and the results were compared with each other.

DNA-denaturation

Due to positive results of cytotoxicity against tumor cells, evaluation of Pd(II) complex interaction with DNA in the absence (i) and presence of vitamin K3 (ii) was done.

The UV-Vis spectrum analysis on the interaction of DNA with Pd(II) complex in the absence of VK3 was shown in Fig. 3A. UV spectrum of DNA is very sensitive to conformation changes because regular stacking of the nucleotide chromophores in helical structures leads to the phenomenon of hypochromicity [21], while the red (bathochromic) shift has been associated with the decrease in the energy gap between the HOMO and LUMO molecular orbitals after binding of the complex to DNA. After interaction of Pd(II) complex with DNA, the UV spectrum of Pd(II) complex has 5 nm bathochromic shift at 260 nm. Thus, Fig. 3 could be an evidence for the interaction and probably intercalation of Pd(II) complex with DNA duplex.

In the next step, UV-Vis spectrum of DNA interaction with Pd(II) complex in the presence of VK3 was recorded. Wang and co-workers showed that VK3 can intercalate in to double helix of DNA [22], thus now we have two intercalators in the solution (Pd complex and VK3). UV

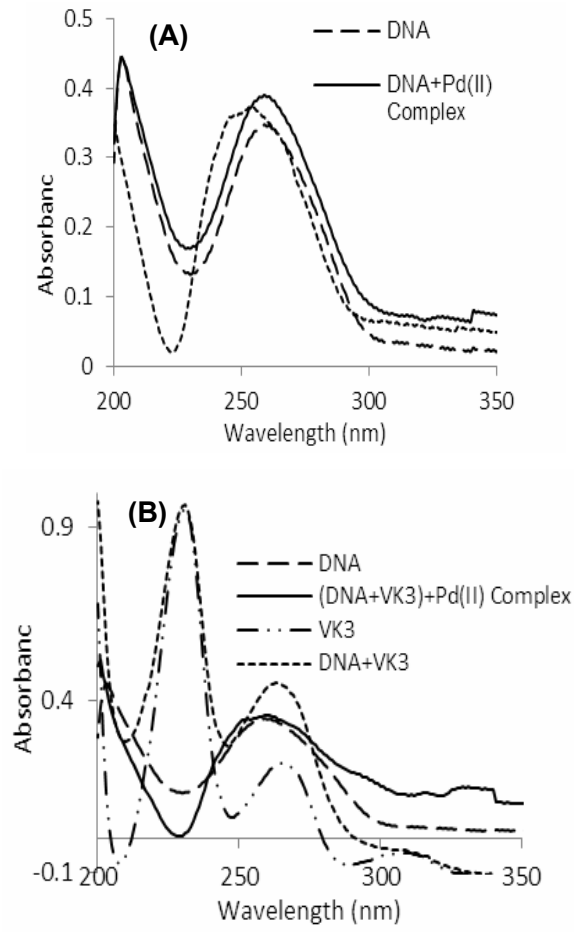

Fig. 3. The absorption spectra of (A) Pd(II) complex, DNA and Pd(II) complex-DNA system and (B) vitamin K3, DNA, VK3-DNA and Pd(II) complex-(VK3+DNA) systems at pH 7.4.

Table 1. Thermodynamic Parameters and Values of $L_{1/2}$ of DNA Denaturation by [Pd(phen)(py-dtc)]NO_3Complex in the Absence (i) and Presence (ii) of Vitamin K3

Compound	T (° C)	$L_{1/2}$	m (kJ mol^{-1}) $(mM)^{-1}$	$\Delta G°_{(H2O)}$ (kJ mol^{-1})	$\Delta H°_{(H2O)}$ (kJ mol^{-1} K^{-1})	$\Delta S°_{(H2O)}$ (kJ mol^{-1})
	27	0.046	275.1	14.88		0.068
(i)	37	0.044	375.5	14.19	35.42	0.068
	27	0.026	341.0	14.88		0.067
(ii)	37	0.025	444.6	14.20	35.13	0.067

spectra of Pd(II) complex-DNA interaction in the presence of VK3 were shown in Fig. 3B. The bathochromic shift in this spectrum is 2 nm more than that of in Pd(II) complex-DNA system. Therefore, VK3 can be affect the binding of Pd(II) complex to DNA.

To further explore, denaturation experiments of CT-

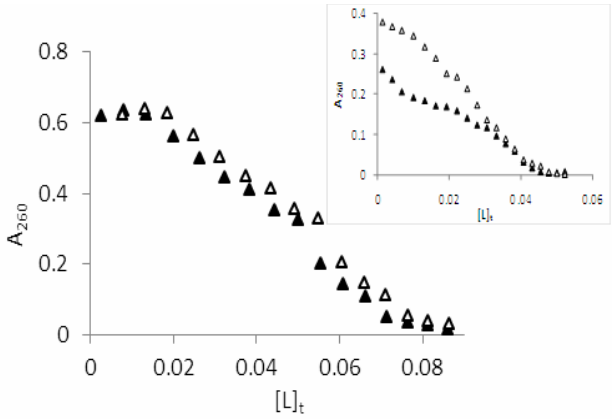

Fig. 4. The absorbance changes of DNA at $\lambda_{max} = 260$ nm due to increasing the total concentration of $[Pd(phen)(py\text{-}dtc)]NO_3$ at 27 (Δ) and 37(▲) °C (Insert for $[Pd(phen)(py\text{-}dtc)]NO_3$ in the presence of vitamin K3).

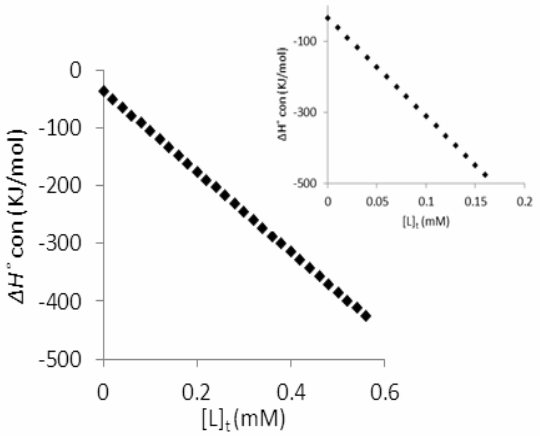

Fig. 5. Plots of the molar enthalpies of CT-DNA denaturation in the interaction with Pd(II) complex and the insert for $[Pd(phen)(py\text{-}dtc)]NO_3$ in the presence of vitamin K3 in the range of 27-37 °C.

DNA with increasing of Pd(II) complex in the absence and presence of VK3 were performed (Fig. 4). The concentration of the metal complex in the midpoint of transition, $[L]_{1/2}$, were obtained 0.046 mM and 0.044 mM in 27 and 37 °C, respectively. In general for DNA denaturation, the side effects would be less if a lower concentration of drug to be used. In the presence of VK3 values of $[L]_{1/2}$ reduced to 0.026 mM and 0.025 mM (Table 1). Thus, if Pd(II) complex is used as an anti-cancer drug in combination with VK3, will have fewer side effects.

The interaction of CT-DNA with alone VK3 was also performed in which by increasing vitamin, the changes of absorbance at 260 nm was limited. However, the interaction of VK3 and DNA is not too strong to be denatured the CT-DNA, but can slightly alter the structure of nucleic acid. These changes will facilitate the DNA denaturation by means of Pd(II) complex.

Thermodynamic parameters of above interactions using the DNA denaturation plots (Fig. 4) and the Pace method were calculated (Table 1). Here, ΔG_{H2O} is conformational stability of DNA in the absence of metal complex and m is a measure of the metal complex ability to denature DNA. The values of m for Pd(II) complex + VK3 system are higher than those of alone Pd(II) complex which indicate the

higher ability of Pd(II) complex + VK3 system to denature DNA. As we know, the higher the value of ΔG°, the larger the conformational stability of DNA. However, the values of ΔG° decrease by increasing the temperature for both cases. This is based on expectations because in general, most of the macromolecules are less stable at higher temperature.

Molar enthalpy of CT-DNA denaturation in the absence of Pd(II) complex, ΔG_{H2O}, is another important thermodynamic parameter. To found this, we calculated the molar enthalpy of CT-DNA denaturation in the presence of the Pd(II) complex and Pd(II) complex + VK3 system at 27 and 37 °C using Gibbs-Helmholtz equation [23]. On plotting the values of these enthalpies versus the concentration of metal complex, straight lines will be obtained which are shown in Fig. 5 for Pd(II) complex and the insert for Pd(II) complex + VK3 system. Intrapolation of these lines gives the values of ΔG_{H2O} (Table 1). These plots show that in the range of 27-37 °C the changes in the enthalpies in the presence of Pd(II) and Pd(II) complex + VK3 system are descending. These observations indicate that, on increasing the concentration of Pd(II) and Pd(II) complex + VK3, the stability of CT-DNA is decreased. Moreover, the entropy, ΔS_{H2O} , of DNA unfolding by Pd(II) complex has been calculated using equation $\Delta G° = \Delta H° - T\Delta S°$. These data show that the Pd(II) complex-DNA system is more disordered than that of native DNA, because the entropy changes are positive for Pd(II)- or VK3+Pd(II)-DNA complexes in the denaturation processes of CT-DNA.

Absorption Titration Measurements and Binding Parameters

The interaction of above complex and DNA was characterized classically through absorption titrations. Solution containing a constant concentration of complex in the absence (i) and presence of VK3 (ii) was titrated with increasing amounts of DNA, and the absorption spectra were recorded. The maximum ΔA (ΔA_{max}) of the metal complex totally bound to DNA was determined by intrapolation of a plot of reciprocal of ΔAs against the reciprocal of [DNA] obtained from each DNA concentration (Table 2).

In the next experiment, titration of fixed amount of DNA with variation of equal amounts of the complex and complex + VK3, the concentration of the complex bound to DNA, $[L]_b$, was obtained. Then, values of ν, that is, the ratio of the concentration of bound metal complex to the total DNA was calculated. The Scatchard plots were obtained separately for two modes i and ii at 27 and 37 °C by plotting $\nu/[L]_f$ *vs*. ν [24] (Fig. 6). Both plots are curvilinear concave downward, suggesting cooperative binding for both modes i and ii [24].

To obtain the binding parameters, the above experimental data (ν and $[L]_f$) were substituted in Hill equation, $\nu = g(K[L]_f)^n/(1 + (K[L]_f)^n)$ [25]. This equation contains three unknown parameters n, K, and g, where n is the Hill coefficient (n = 1 indicates noncooperative, n > 1is cooperative, and n < 1 shows anticooperative binding of DNA with metal complex), g is the number of binding sites per 1000 nucleotides of DNA and K is the apparent binding constant. Using Eureka software, the theoretical values of these parameters was deduced (Table 2). In both modes (i) and (ii) the values of n indicates that the Pd(II) complex binds to DNA cooperatively and binding at one site increases the affinity of binding at the other sites. Here we have a series of five binding sites and VK3 does not have significant effect on the number of binding sites. The apparent binding constants of the DNA-Pd(II) complex interaction are increased with increasing of VK3. This suggests that vitamin K3 can be reinforces the tendency of palladium(II) complex to interactions with DNA. As mentioned in the previous section, main reason for the increase in K values in the presence of VK3 may be caused by conformational changes in DNA by VK3 which is led to the strengthening of the interaction between the Pd(II) complex and DNA.

Fluorescence Spectroscopic Studies

No fluorescence was observed for the above palladium(II) complex at room temperature in aqueous solution or in the presence of calf thymus DNA. So, the binding of the palladium(II) complex and DNA cannot be directly presented in the emission spectra. Hence, competitive ethidium bromide (EBr) binding studies were undertaken in order to determine the extent of binding of the palladium(II) complex to DNA.

It should be noted that alone vitamin K3 has the fluorescence emission and its intensity was dramatically

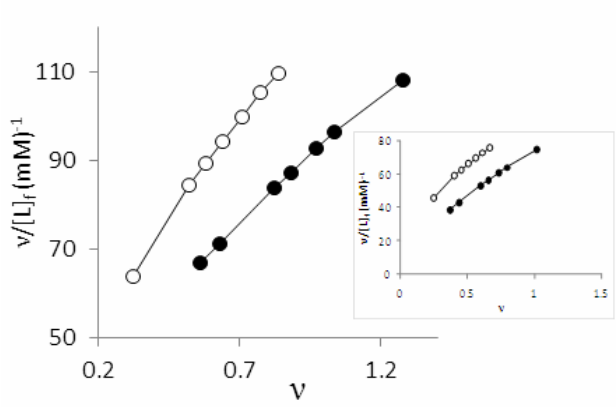

Fig. 6. Scatchard plots for binding of [Pd(phen)(py-dtc)]NO_3 with CT-DNA. The insert is Scatchard plots for binding of [Pd(phen)(py-dtc)]NO_3 with CT-DNA in the presence of vitamin K3 at 27 (○) and 37(●) °C.

Table 2. Values of ΔA_{max} and Binding Parameters in the Hill Equation for Interaction between CT-DNA and [Pd(phen)(py-dtc)]NO_3 Complex in the Absence (i) and Presence (ii) of Vitamin K3 in 20 mM Tris-HCl Buffer and pH 7.4

Compound	T (°C)	ΔA_{max}	g	K (M^{-1})	N	Error[a]
	27	0.301	5	48.84×10^3	3.34	0.001
(i)	37	0.214	5	49.87×10^3	2.27	0.0005
	27	0.329	5	69.92×10^3	2.55	0.0006
(ii)	37	0.291	5	59.48×10^3	3.00	0.001

[a]Maximum error between theoretical and experimental values of ν.

enhanced when the DNA was added, which indicated that vitamin K3 could bind to DNA [26]. This enhancement in fluorescence emission may be largely due to the increase of the molecular planarity of vitamin K3 and the decrease of the collision frequency of the solvent molecules with vitamin K3, which is caused by the planar aromatic group of vitamin K3 stacks between adjacent base pairs of DNA. In other words, DNA protected vitamin K3 from the quenching effect of the solvent molecules, so the emission intensity of vitamin K3 increased noticeably [26]. Figure 7A shows the emission spectra of DNA-EBr system in the absence and presence of different concentrations of the alone Pd(II) complex at 27 °C. As shown in Fig. 7A, the Pd(II) complex markedly reduces the intrinsic fluorescence emission of DNA-EBr system at different concentrations and quenches it. So, it can be concluded that the Pd(II) complex binds to DNA [25]. This experiment was performed in the presence of equal concentration of vitamin K3 and Pd(II) complex. Expected in this case, that two molecules (Pd complex and VK3) are competing with ethidium bromide for interaction with DNA, the intensity of the fluorescence emission of EBr-DNA system, decreases with more intensity that is partly observable in Fig. 7B.

To further explore the mechanism of fluorescence quenching was evaluated. Fluorescence quenching can occur by different mechanisms, which usually classified as dynamic quenching and static quenching. Dynamic quenching refers to a process that the fluorophore and the quencher come into contact during the transient existence of the excited state, whereas static quenching resulting from the formation of a ground state complex between the fluorophore and the quencher. Static and dynamic

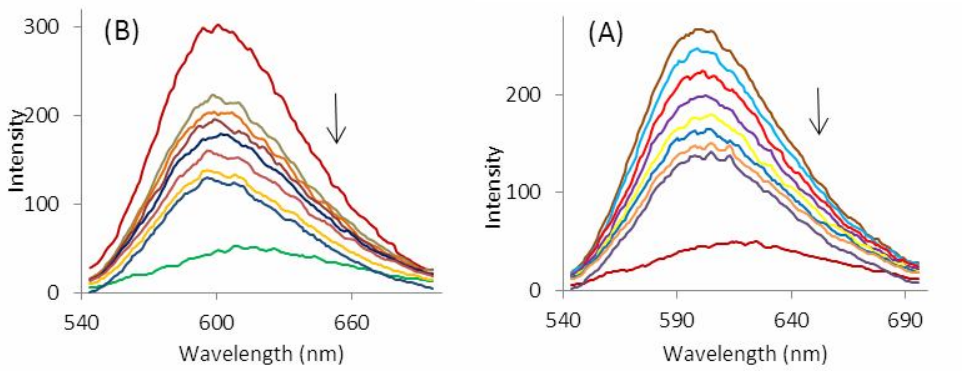

Fig. 7. Fluorescence emission spectra of interacted EBr-DNA in the presence of different concentrations of Pd(II) complex and the inset for Pd(II)complex + VK3 at pH 7.4.

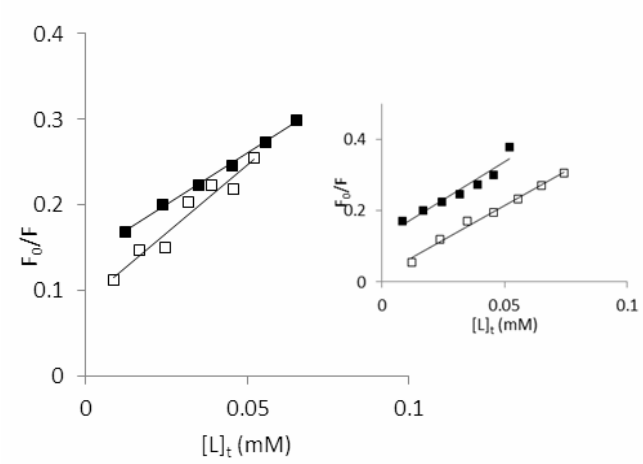

Fig. 8. Stern-Volmer plots of fluorescence quenching of the Pd(II) complex-DNA system at 27 (□) and 37 (■), insert for Pd(II)complex in the presence of vitamin K3, pH 7.4.

Table 3. Stern-Volmer Quenching and Binding Constants and Number of Binding Sites for the Interaction of Pd(II) Complex with DNA in the Absence (i) and Presence (ii) of Vitamin K3

Compound	T (°C)	K_{sv} (M^{-1})	[a]R	K_b (M^{-1})	N	[a]R
(i)	27	3.11×10^5	0.9987	5.33×10^5	1.021	0.9833
	37	1.98×10^5	0.9956	4.67×10^5	1.321	0.9911
(ii)	27	11.43×10^6	0.9976	8.04×10^6	0.991	0.9951
	37	9.03×10^6	0.9982	6.88×10^6	1.034	0.9816

[a]R is the correlation coefficient related to K_{SV}. [b]R is the correlation coefficient related to K_b.

quenching can be distinguished by their different binding constants dependence on temperature and viscosity, or preferably by lifetime measurements [17]. In this paper, we have used the binding constants dependence on the temperature to elucidate the quenching mechanism. Fluorescence quenching data were analyzed through the Stern-Volmer equation:

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

Here, F_0 and F are the fluorescence intensities of DNA in the absence and in the presence of Pd(II) complex, respectively, [Q] the concentration of quenching reagent and K_{SV} is the Stern-Volmer constant.

The values of K_{SV}, for above Pd(II) complex has been determined from the plot of F_0/F *vs*. [DNA] in the absence and presence of VK3 at 27 and 37 °C (Fig. 8). The results (Table 3) showed that in the absence and presence of VK3 the Stern-Volmer quenching constants were inversely correlated with temperature which indicated that the quenching mechanism of DNA-EBr by Pd(II) complex was most likely a static quenching procedure. This means that the increasing of temperature will decrease the stability of the ground state complex, which will cause the fluorescence quenching decreasing. On the other hand, vitamin K3 increases the fluorescence quenching of DNA-EBr system by pd(II) complex but has no significant effect on kind of this mechanism.

When small molecules binding independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation [26,27]:

$$\log(F_0 - F)/F = \log K_b + n \log[Q] \quad (2)$$

Here, F_0 and F are the florescence intensity without and with the Pd(II) complex, respectively [28]. A plot of $\log[(F_0 - F)/F]$ *vs*. log[Q] gave a straight line using least squares analysis whose slope was equal to n (binding site number) and the intercept on Y-axis was $\log K_b$ (K_b = binding constant). This plot has been shown in Fig. 9 for Pd(II) complex and the insert for Pd(II) complex + VK3 system that indicates a simple binding process for the complex in both modes. K_b and n were reported in Table 3. These results is consistent with the results of Stern-Volmer and confirms that the fluorescence quenching mechanism of DNA-EBr system by pd(II) complex is static and VK3 as a contributing factor may facilitate the fluorescence quenching. Here, based on the k_q and K_b values, the following result can be suggested: (i) With respect to the strong drug-bio macromolecules systems which their corresponding binding constants have been reported from 10^5 to 10^6 M^{-1}, the values of binding constant between the Pd(II) complex and DNA confirm the existence of strong interactions [32,33] (ii) Compared with the compounds used in the clinical phase (with K_b about 10^4-10^6 M^{-1} such as doxorubicin and cisplatin) the probability of binding formation between Pd(II) complex and DNA at situation *in vivo* is predictable (iii) Groove binding unlike intercalation, does not induce large conformational changes in DNA and may be considered similar to standard lock-and-key models for ligand-macromolecular binding. Groove binders are stabilized by intermolecular interactions and typically have larger association constants than intercalators (approximately 10^{11} M^{-1}). So, due to the K_b values and blue shift in UV-Vis spectra of DNA (Fig. 3A) the interaction of Pd(II) complex with the purine and pyrimidine bases of DNA *via* intercalation is predictable [32].

Further studies to characterize the mode of binding of $[Pd(phen)(py\text{-}dtc)]NO_3$ to DNA in the absence and presence of VK3 were carried out. The number of EBr molecules intercalated to DNA in the presence of different concentrations of the Pd(II) complex was calculated using Scatchard analysis [29]. As shown in Fig. 10A and Table 4, Pd(II) complex inhibits competitively the EBr binding to CT-DNA (type-A behavior) [30], where number of binding sites n, (intercept on the abscissa) remain constant and the slope of the graphs, K_{app}, (apparent association constant) decreases with increasing the concentration of Pd(II) complex. This implies that the $[Pd(phen)(py\text{-}dtc)]NO_3$ complex is intercalating in CT-DNA and thereby competing for intercalation sites occupied by EBr. This experiment was repeated in the presence of Pd(II) complex and vitamin K3 (Fig. 10B). Figure 10B shows that with increasing concentration of the Pd(II) complex and vitamin K3 (with ratio of 1:1), values of n and K are changed (type-B behavior). Generally, the variation of n and K suggests a mixed interaction. Wang and colleagues demonstrated that

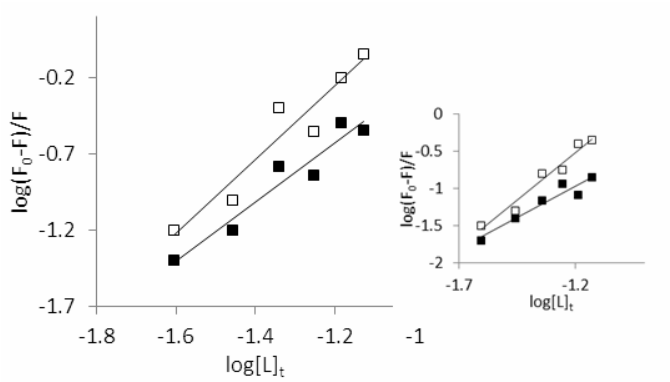

Fig. 9. The plots of log (F_0- F)/F *vs*. log[L] at two different temperatures for Pd(II) complex-DNA system and insert for Pd(II)complex-DNA in the presence of vitamin K3.

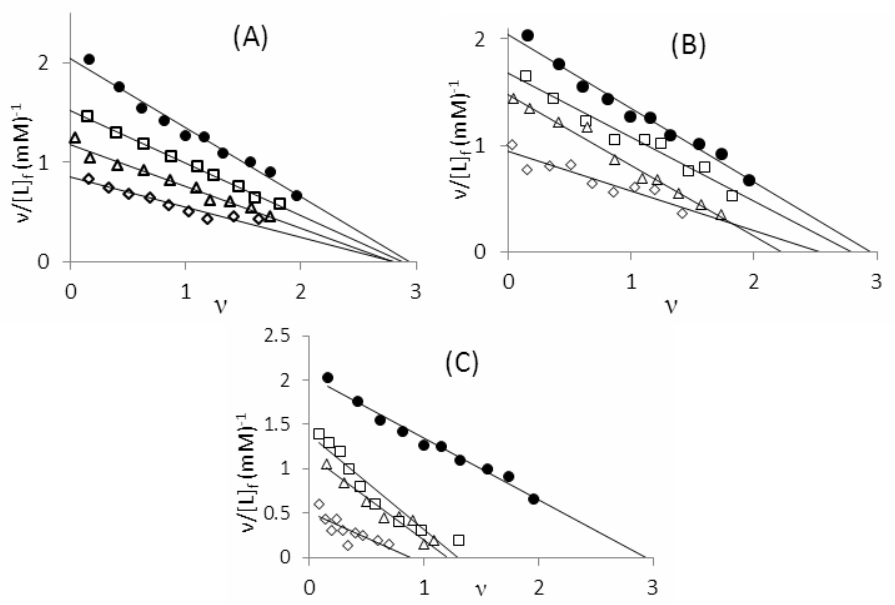

Fig. 10. Competition between ethidium bromide with (A) Pd(II)complex (B) Pd(II) complex + vitamin K3 (1:1) (C) alone vitamin K3 for the binding sites of DNA (Scatchard plot). Scatchard's plot was obtained with 60 µM calf thymus DNA (●). 10 µM (□), 20 µM (Δ) and 30 µM (◊) for Pd(II) complex, Pd(II) + vitamin K3 and alone vitamin K3 were added in the presence of increased concentrations of EBr (2, 4, ... , 20 µM) (pH 7.4).

the modes of interaction between vitamin K3 and DNA are intercalation and static electronic effects [31] (Fig. 10C). Thus the change of Scatchard plot for pd(II) complex in the presence of VK3, is predictable. As can be seen in Table 4 the values of n for interaction of Pd(II) complex with DNA-EBr system in the presence of VK3 slightly decreased which shows the competition between [Pd(phen)(py-dtc)]NO_3 complex and VK3 for interaction with DNA-EBr system. In this interaction K_{app} in the presence of VK3 was increased which could be confirmed the UV-Vis results.

Table 4. Binding Parameters for [Pd(phen)(py-dtc)]NO_3 Complex in the Absence (i) and Presence (ii) of Vitamin K3 and Alone Vitamin K3 (iii) on the Fluorescence of EBr-DNA System

Compound	r_f^a	K (M^{-1})	N
	0.00	0.694×10^5	
	0.50	0.527×10^5	
(i)	1.00	0.419×10^5	2.90×10^{-3}
	1.50	0.303×10^5	
	0.00	0.694×10^5	2.90×10^{-3}
	0.50	0.503×10^5	2.75×10^{-3}
(ii)	1.00	0.665×10^5	2.20×10^{-3}
	1.50	0.373×10^5	2.55×10^{-3}
	0.00	0.694×10^5	2.90×10^{-3}
(iii)	0.50	1.072×10^5	1.35×10^{-3}
	1.00	0.952×10^5	1.19×10^{-3}
	1.50	0.580×10^5	0.85×10^{-3}

[a]Formal ratio of metal complex to nucleotide concentration.

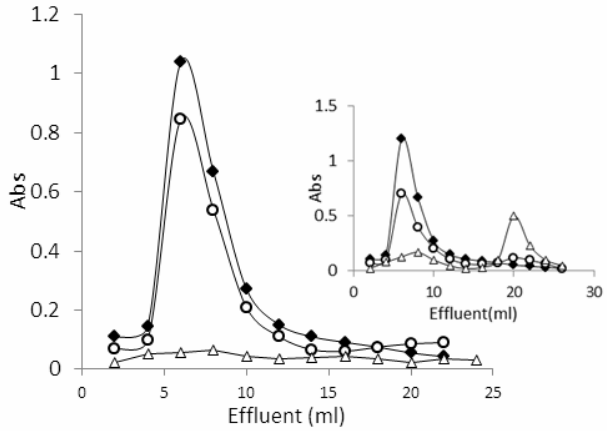

Fig. 11. Gel chromatograms related to DNA interaction with Pd(II) complex at 260 nm (♦), 302 nm (○) and 441 nm (Δ), (The insert for Pd(II) complex + vitamin K3) obtained on Sephadex G-25 column, equilibrated with 20 mM Tris-HCl buffer of pH 7.4 in the presence of 20 mM sodium chloride.

Binding Modes

The mode of binding between CT-DNA and the above Pd(II) complex in the presence and absence of VK3 was further investigated by gel filtration experiment. In this experiment, the solutions of DNA-Pd(II) complex and DNA-Pd(II) complex-VK3 (with ratio 1:1 of metal complex and VK3) were passed through a Sephadex G-25 column equilibrated with Tris-HCl buffer separately. Elution was done with buffer and each fraction of the column was monitored spectrophotometrically at 303 nm, 260 nm and 441 nm (related to metal complex, DNA and VK3). The gel chromatograms obtained from these experiments are given in Figs. 11. These results show that in the absence of VK3 the two peaks obtained at two wavelengths (303 nm and 260

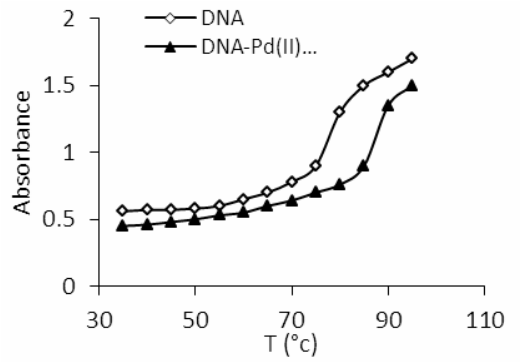

Fig. 12. Temperature profile at 260 nm for DNA in Tris-HCl buffer and DNA plus Pd(II) complex. As DNA interactions with Pd(II) complex stabilize the DNA duplex, a characteristic increase in the T_m is observed in the presence of a DNA-binding compound upon thermal denaturation.

nm) were not clearly resolved in Fig. 11, which indicates that metal complex was not separated from DNA and their binding with DNA is strong enough not to break readily.

In the case of DNA-Pd(II) complex-VK3 system, metal complex was not separated from DNA but two peaks were observed for wavelength of 441 nm (related to VK3). The left peak is smaller than the right and observed along with two other peaks (303 nm and 260 nm) which indicate that limited amount of VK3 bound to the CT-DNA (or metal complex) and the remainder without any chemical bond with DNA, are removed from the column. So compared with the palladium complex, a limited number of VK3 molecules have strong bonds with CT-DNA.

Thermal Denaturation

For further investigation, DNA thermal denaturation studies can be performed. In these experiments duplex DNA is thermally denatured into single-strand components in the presence and absence of Pd(II) complex. The melting profile of DNA was sensitive to the addition of Pd(II) complex as shown in Fig. 12. The upper curve, the DNA control in Tris-HCl buffer, has a T_m of 79 °C; addition of Pd(II) complex to DNA solution increases the T_m to 90 °C. The increase of 11 °C of the melting temperature of DNA indicates that intercalation binding interaction between the stacked Pd(II) complex and DNA strongly stabilizes the conformation of DNA [32].

CONCLUSIONS

In this work, a new potential antitumor agent, $[Pd(phen)(py\text{-}dtc)]NO_3$ has been synthesized and characterized. This complex is especially active against chronic myelogenous leukemia K562 cell lines and significantly its toxic properties increases in the presence of Vitamin K3. Detailed analysis of the binding of above metal complex with CT-DNA by fluorescence, UV-Vis techniques and gel chromatography are carried out. This complex in the presence of VK3 unexpectedly denatures CT-DNA at very low concentration. Several binding parameters are also presented. This complex quenched the emission intensity of the DNA-EBr system mainly by static quenching. Experimental results suggested that in the absence and presence of VK3, the mode of intercalation might play major role in the interactions of Pd(II) complex with DNA. In conclusion we hope that these two drugs worked together to kill cancer cells, supporting each other whereas the least side effects are shown. Of course, more research is necessary in order to improve anticancer treatments and to decrease toxic effects.

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