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Synthesis, Characterization, Anticancer, DNA Binding and Antioxidant Studies of Benzylamine Supported Pd (II) Complex

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Abstract

A benzylamine supported palladium (II) complex has been synthesized and characterized using different spectroscopic techniques, such as FTIR, UV/Vis., ^{1}H, ^{13}C NMR and LCMS. The semi crystalline nature of the complex was investigated through powder XRD and SAED pattern analysed by HRTEM. The *in vitro* biological study against breast cancer cell lines MCF-7 and MDA-MB-231 was not much effective but their strong DNA binding activity (DBA) compared to free ligand suggested them as a good DNA binder and makes them interesting against other cancerous cell lines. The DBA further supported by physicochemical studies such as surface tension and viscosity of complex+DNA which inferred the disruption and intercalation of DNA and complex. The scavenging activity (SA) also proves the anticancer nature of complex, in this context, the scavenging activity of complex was determined through DPPH• free radical and the result indicates good antioxidant behaviour of complexes.

Keywords: Cisplatin; Anticancer; Breast cancer; SAR; DBA; Binding constant; Antioxidant

Introduction

From last few decades the breast cancer is one of the major issues in health care in terms of morbidity, mortality and therapy costs [1]. In addition, although many more drugs have been introduced into the market but the response to therapy is still poor. Therefore, there is an urgent need for more efficient anticancer drugs to be developed. The foremost target of most research groups is to find a convenient anticancer drug that can be used efficiently for the treatment of human tumours. In recent years, numerous Pd (II) complexes with promising activity against tumour cell lines have been synthesized and published [2,3]. Importantly, a good relationship was observed between the cytotoxic activity of the Pd complexes and their lypophilicity or solubility [4]. In fact, Pd complexes, as non-platinum complexes, have recently been reviewed to have a significant anti-tumour activity to cancer cells as well as lower side effects compared to cisplatin such as nephrotoxicity, drug resistance, renal and cervical problems [5-7]. As an important feature of metal-containing anti-cancer agents, Pd complexes are expected to have less kidney toxicity than cisplatin [8]. Earlier studies reported that Pd complexes demonstrated significant anti-tumour activity comparable with cisplatin [9]. Further to that a new Pd complex demonstrating potent cytotoxic activity against different cancer cell lines has recently been reported [10-12]. Better solubility of Pd complexes, compared to platinum, seems to make Pd complexes more attractive. In one study, Pd complexes of glyoxylicoxime were found to have higher aqueous solubility than Pt (II) complexes of glyoxylicoxime [13]. In this context the discovery of non-platinum based biological complexes came into consideration [5-7]. The development of palladium anticancer drugs has not been promising, probably because their design has been based on structure–activity considerations generated from platinum antitumor drugs. Bearing in mind that Pd (II) complexes are about 105 times more reactive than their Pt (II) analogues, the low antitumor activity of Pd compounds has been attributed to very rapid hydrolysis of the leaving groups that dissociate readily in solution, leading to reactive species far from their pharmacological targets [14,15]. Palladium is a suitable candidate for metallo-drugs because it displays structural properties similar to those of platinum and also exhibits promising cytotoxicity. As part of our continuing interest in the synthesis of transition metal complexes of biological molecules, we have investigated the coordination behaviour of Pd (II) complex with benzylamine ligand and analyzed there in vitro antitumor activity against different breast cancer cell lines. Since, DNA is a primary molecular target of anticancer drugs, and ascertains an extent of a drug's chemotherapeutic potential, thus alongside anticancer activity, the DBA of synthesized complex have chosen to investigate their anticancer nature [16]. Apart from DNA binding, the antioxidant activity has also been proven the anticancer nature of the Pd (II) complex [17], which have been the criteria for analyzing their antioxidant property. Therefore, the biological study of Pd (II) complex leads to a better understanding of their biological aspects, especially, in oncology research and attempts to opens new avenues about activities. Hence, our study is an attempt to get overcome from such problems up to some extent. So, with the aforesaid objectives we have synthesized new Pd (II) complex with Benzylamine and analysed their in vitro anticancer activity, DNA binding and scavenging activity.

Results and Discussion

Synthesis

PdBA has been synthesized allowing reaction of K_2 $PdCl_4$ with different benzylamine (BA) in (1: 2) ethanol+water solution over 24 h as per reaction schemes.

Reaction scheme: Synthetic route of PdBA: The 3300 to 3119 cm^{-1} stretching frequencies inferred presence of NH2 of benzylamine in the complexes and similarly from 1497 to 1453 cm^{-1} predicted C=C in

phenyl ring. The 495.92 to 438.78 cm^{-1} [18-20] has confirmed Pd–N coordinate bands and 380-348 cm^{-1} indicates Pd–Cl bands. In 1H NMR, the 2H of -NH2 and PhCH2- appeared at δ 3.93 to 2.086 and 5.17 to 3.85 with singlet for the synthesized complex. In complex, the 2H of C3 and C7 showed a doublet at δ 7.29-7.28 with J=6.5 MHz where a triplet of 2H of C4 and C6 was found at δ 7.45-7.47 having J= 7.3 MHz. A multiplet of H of C5 was found at δ 7.49-7.51. In 13C NMR, the benzyl carbon C1 at δ 47.84, C2 at 138.34, C3 and C7 found at 127.57-127.14, C4 and C6 found between 128.73-128.36 and C5 found at 128.1 (ESI† Figures S1 and S2) [21,22]. The +ve ESI mass spectra of Pd complex have found [M+1] for complex confirming its molecular mass. To investigate a solid state structure retained in solution, the UV/ Vis spectral behaviour was investigated in DMSO, DMSO+water as well as in DMSO+phosphate buffer for the complex (Figures 1-3). The overall patterns of spectra for complexes solution were found similar with the different mediums to ensure their sustainability in liquid state. The UV/Vis absorption from 265 to 270 nm and 1H NMR coupling constant between 5 and 9 MHz, have confirmed their trans geometry [18,23,24].

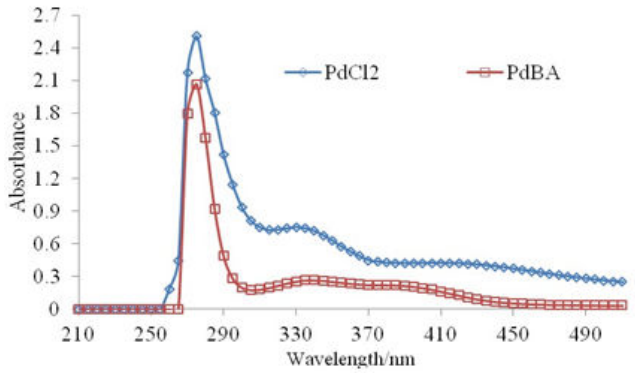

Figure 1: UV Spectra of PdBA in DMSO at 0.001M.

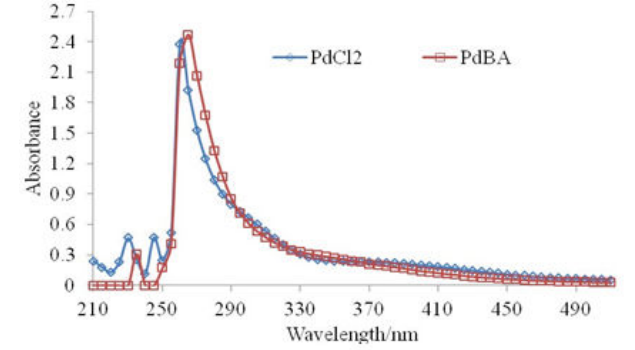

Figure 2: UV Spectra of PdBA in DMSO + water at 0.001M.

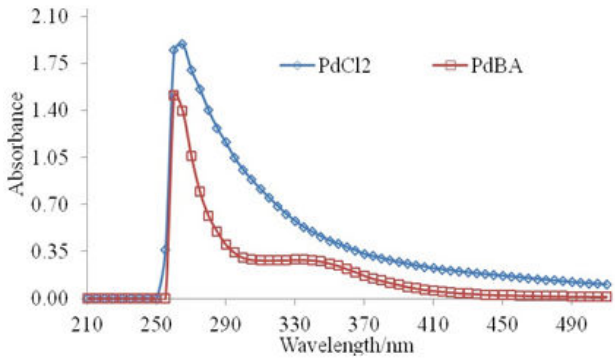

Figure 3: UV Spectra of PdBA in DMSO + PBS (7.2 pH) at 0.001M.

XRD and SAED analysis of the complex

XRD powder pattern peaks for PdBA are shown in Supplementary material indicate the semi crystalline nature of the compound. The highest intensity peak was evaluated at 7.982θ angle with 11.04938 Å d-spacing values. The full width half-maximum (FWHM) value for this peak was 0.205. The semi crystalline nature of the complex was investigated by using XRD data indicates that the PdBA are semi crystalline in nature (ESI† Table 1 and Figures S3). To support the XRD data the more advance SAED pattern of the PdBA investigated with HRTEM by dispersing the PdBA in water and dry up to absolute dryness. The TEM images reports from the SAED pattern was taken at different magnification suggesting the presence of different elements which are semi crystalline and homogeneous in shape and size (ESI† Figures S4). The proper alignment is not seen in the rings which indicate that the complex is not completely crystalline but a clear formation of rings suggesting the semi crystalline nature of complexes.

Anticancer activity

The complexes were tested *in vitro* against MCF-7 and MDA-MB-231 human breast tumour cell lines by colorimetric micro culture 2-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-5-sulfo-benzenesulfonate (SRB) assay [25], and compared with adriamycin (ADR) and cisplatin [26]. The MCF-7 and MDA-MB-231 interaction activities for 10, 20, 40 and 80 μg mL^{-1} PdBA have been illustrated (Figure 4a and 4b). The analyzed GI50, TGI and LC50 in μg mL^{-1} have inferred 50% growth inhibition, resultant total growth inhibition and a net loss of 50% cells after treatment respectively (Table 1). The GI50 less than 10 μg mL-1 depicts anticancer activity with respect to ADR and cisplatin [25,26]. The anticancer activity of PdBA (GI50>10) as compared to standard ADR and cisplatin those have GI50 values<10 indicates less anticancer activity (Table 1).

The GI50 values for complex are not much closer to standards, listing them non anticancer but are closer to LC50 causing loss of 50% cancerous cells. The complex has been synthesized for the treatment of cancer but our focus was to investigate the anticancer activity of complex with benzylamine as ligands on breast cancer [26].

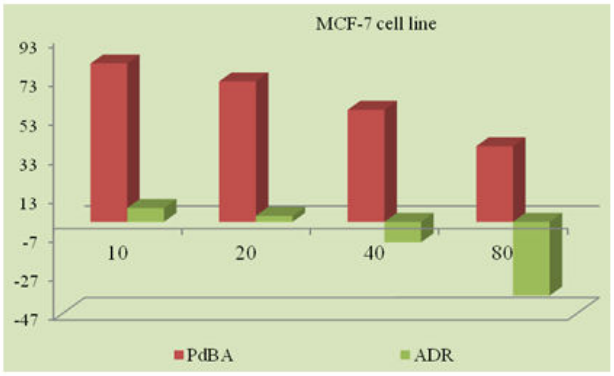

Figure 4a: Growth curve: Human breast cancer cell line MCF-7.

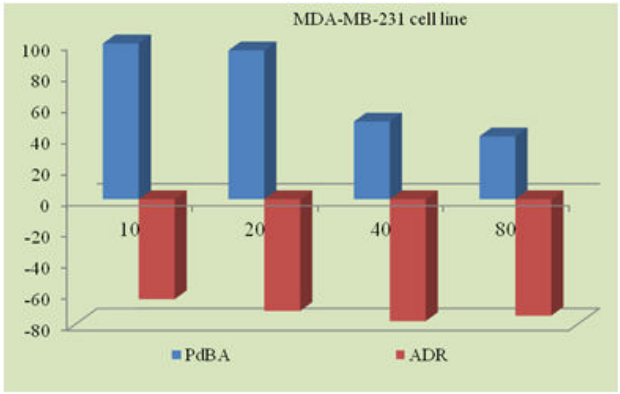

Figure 4b: Growth curve: Human breast cancer cell line MBA-MB-231.

Entry	Complexes	MCF7			MDA-MB-231		
		LC50	TGI	GI50	LC50	TGI	GI50
1	PdBA	>80.0	>80.0	59.3	>80.0	>80.0	62.6
2	ADR	79.2	40.5	<10.0	39.85	<10.0	<10.0
3	Cisplatin33	>30.0	>30	<10.0	>30.0	>30.0	<10.0

Table 1: LC, TGI and GI values (μg/mL) against MCF7 and MDA-MB-231 cell lines of complexes, an anticancer analysis.

DNA binding activity

Through spectrophotometric study: DBA has explained the anticancer nature of the complex or the drug, analysed with absorption spectral study. In light of this the PdBA were mixed with CT-DNA separately at certain concentration resulted in changes in absorbance. Generally, the hypochromism effect in UV study reveals their intercalative binding strength attributed to an interaction with DNA bases [27,28]. Similarly, a hyperchromic effect ascribed to an external contact or to a partial uncoiling of DNA structure, exposing more bases of DNA may be due to electrostatic binding [29,30]. Such observations have been noticed in present study where a significant hypochromic effect in absorption titrations of DNA with PdBA exposed an intercalation with the base pairs of DNA [28,31] (Figure 5a). The decrease in UV absorption predicted the stronger interactions of PdBA whose intercalating strengths was dependent on size and electron densities of interacting aromatic rings as side arm with an amine group [27-30]. Through UV spectrophotometric titration the PdBA+DNA binding constant (Kb) were 1 × 104 M2 cm for PdBA has been reported. The calculated Kb values for PdBA are 1 × 104 M^{-1}, a lower magnitude than that of the classical intercalator EB (Ethidium bromide) (Kb=1.23 (± 0.07) × 105 M^{-1}) and both reveal a strong binding to CT-DNA. Their DBA have been stronger in comparison to a free ligand like benzylamine (Figure 5b).

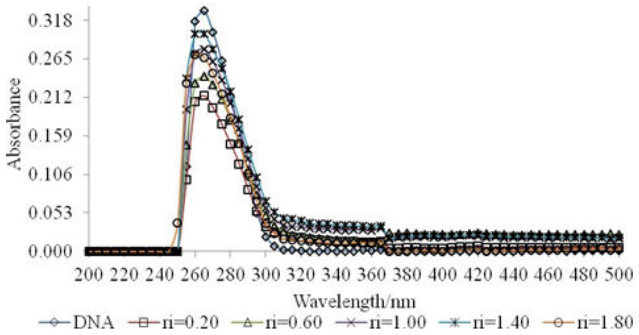

Figure 5a: Absorption spectra of DNA (5 × 10^{-5} M) in absence and presence of increasing amounts of PdBA (ri=[complex]/[DNA]).

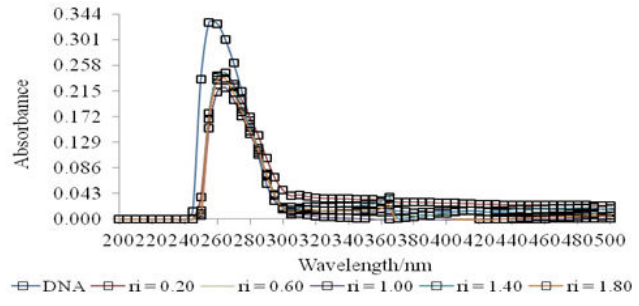

Figure 5b: Absorption spectra of DNA (5 × 10-5 M) in absence and presence of increasing amounts of benzylamine (ri = [complex]/[DNA]).

Through physicochemical study: To support the DBA with spectroscopic study, the viscosity, surface tension, conductivity and zeta potentials are also reported for better understanding.

Viscosity: For a classical intercalative mode, the viscosity is increased of the DNA solution due to an increase in overall DNA length while it is decreased for a partial non-classical intercalation

process causing a bend or kink in the DNA helix that reduces its effective length concomitantly [31,32]. Instance, a decrease in relative viscosity with cisplatin, is explained due to covalent binding and shortening in an axial length of double helix DNA. On the other hand a classical organic intercalator such as ethidium bromide increased the relative viscosity on increasing an axial length of the DNA [33-35]. To observe covalent binding or classical organic interaction, their relative specific viscosities $(\eta/\eta 0)1/3$ ($\eta 0$ and η being specific viscosity contributions of DNA with and without complexes, respectively) were plotted against 1/R (R=[DNA]/[complex]=0.2, 0.6, 1.0, 1.4, 1.8) (Figure 6).

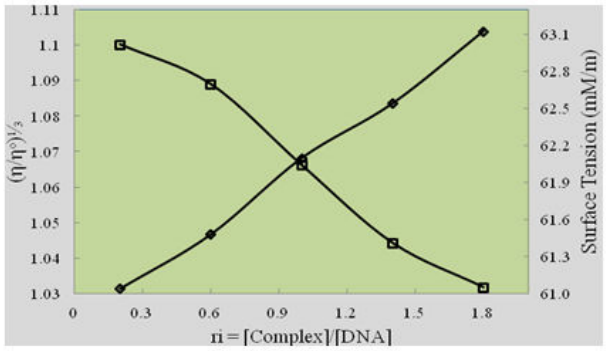

Figure 6: Effect of increasing amounts of PdBA on viscosity and surface tension of CT-DNA (5×10^{-5} M).

On increasing concentration of PdBA, an increased in viscosity of DNA has observed (Figure 6), proving the PdBA as DNA intercalators. For ethidium bromide, the relative viscosity of DNA is increased with a slope from 0 to 0.9448 [36], whereas with 0.001 the relative viscosity of DNA has increased for PdBA. A larger decrease in slope value, maybe due to a strong intercalation of PdBA with DNA, made DNA longer [26,33] developing more species. The viscosity of free ligands (Benzylamine) with DNA was measured and found lower than the complexes, which inferred that interaction of DNA with free ligands is weaker as compared to complex. Therefore, a higher increase in viscosity at different concentration of PdBA+DNA but lower as compared to EB and free ligands clearly explained the slightly stronger intercalation between complexes and DNA [26,33].

Surface tension: Apart from the viscosity, the surface tension is an interaction probing thermodynamic indicator to depict the disruption of intramolecular forces existing in DNA, and acts as evidence for stronger interactions with higher viscosity. This force disruption is measured as a decrease in surface tension, and therefore the surface tension values of a DNA solution with increasing amounts of PdBA (1/R = 0.2, 0.6, 1.0, 1.4, 1.8) have been determined (Figure 6). A decrease in surface tension of PdBA+DNA inferred the weakening of cohesivity or intramolecular interaction of DNA bases has lost on interaction with complex.

Conductivity and zeta potential: Similarly, the conductivity and zeta potential have also been analysed for PdBA–DNA interactive complexes, as key factors for an analysing conformational behaviour of an isolated DNA chain with the complexes. A DNA solution of 50 µM had 3426 µS conductivity while increased amounts of complex decreased the conductivities (Table 2).

Complex	Conductivity (µS)				
	10 µm	30 µM	50 µM	70 µM	90 µM
50 µM DNA	3426	3426	3426	3426	3426
PdBA	2007	1947	1891	1798	1747
*referred for 50µM DNA					

Table 2: Conductivity (µS) of DNA and DNA + complexes.

DNA molecules are negatively charged due to phosphate groups, but by interaction with PdBA, their negative charge density is decreased due to a positively charged metal that balances the charge density. With complexes, the zeta potential of resultant PdBA+DNA also decreased (Figure 7), that inferred an increase in columbic interaction and the disassociation of counter ions in DNA was restricted.

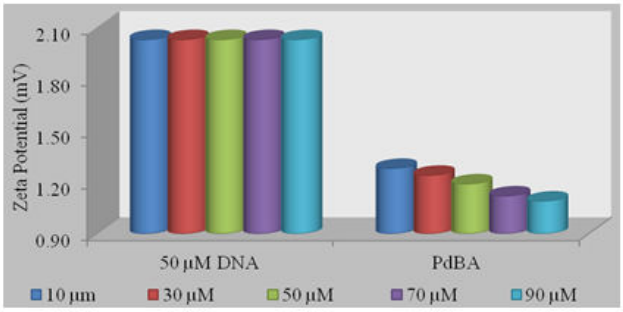

Figure 7: Zeta potential (mV) of pure DNA and DNA + PdBA.

The decrease in conductivity and zeta potential, confirmed that the DNA could structurally be modified due to such interactions.

Drug efficacy studies or DFI with DNA binding

To correlate the DBA with anticancer activity, the DFI has studied [26,33] where the PdBA+DNA interaction have shown their higher viscosities and lower surface tension for PdBA+DNA solution (Figure 6). Thus, a disruption of cohesivity decreases surface tension by developing an interaction between complex and DNA, explained with higher viscosity. Thus, the DFI study attributes complexes as anticancer in nature supported by the DBA of complex [26,33].

Scavenging activities

Antioxidant activities have studied with analysing the decrease in absorbance or scavenging effect of a stable free DPPH• as per standard procedure [37,38]. The percentage scavenging activity of PdBA has been determined with a control of Ascorbic acid in a concentration-dependent mode in comparison to the DPPH• absorption at 517 nm [38-40]. We tried to explain our finding as a consequence of the antioxidant properties of ascorbic acid at low doses and its prooxidant effects with higher doses in comparison of reported metal complexes. The DPPH• free radical's absorption at 517 nm in DMF-water was 0.906. From 50 to 250 µM complexes at an interval of 50 µM, expressed a decrease in absorption (Figure 8) that list them as antioxidant [37,38,40].

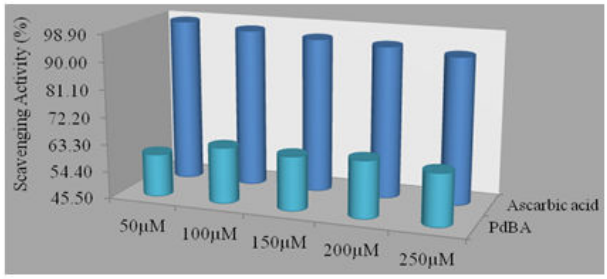

Figure 8: Free radical scavenging activities of synthesized complexes.

The antioxidant activity of PdBA has been lower at 50 µM and highest at 200 µM inferred that the antioxidant potential of the complex at different concentration. For further understanding, the antioxidant potential of complex is also compared with ascorbic acid as a standard. Thus, the antioxidant activity of the PdBA complex has inferred their medicinal significance.

General consideration for synthesis

Initially, K_2PdCl_2 and benzylamine (molar ratio 1:2 respectively) were separately dissolved in freshly prepared solvent (absolute ethanol and Milli-Q water in 1:1.5) using 1 MLH magnetic stirrer. Then, the benzylamine solutions were added drop wise in metal compound solution with continuous stirring at room temperature. After 16 h, the mixture turned from light red brown to greenish, after 24 h, a light greenish colour precipitates were formed. The ppts were filtered off, washed several times with chilled water/ethanol in 1:1 ratio, and kept overnight in vacuum oven at room temperature for absolute dryness.

Characterization data

Synthesis of Bis(phenylmethanamine)dichloropalladium [PdBA]

Yield: 0.1492 g, 67.615%. Elemental analysis, found: C, 42.94; H, 4.63; N, 7.15%. Calcd for $C_{14}H_{16}N_2Cl_2Pd$: C, 43.71; H, 4.71; N, 7.21%.IR (KBr): υmax/cm^{-1} 3273 and 3226 (NH_2), 1497 and 1457 (Ph, C=C), 756.46 (mono substituted Ph), 1075 (C–N), 567.4 (Pd–N), 405.9 (Pd–Cl). 1H NMR (500 MHz; DMSO-d6; Me4Si) δ 2.086 (2H, s, PhCH2NH2), 3.932 (2H, s, PhCH2NH2), 7.28–7.29 (2H, d, PhH, J=6.5 Hz), 7.45–7.47 (2H, t, PhH, J=7.3 Hz) and 7.49-7.51 (1H, m, PhH). 13C NMRs (125 MHz; DMSO-d6; Me4Si) δ 47.601 (C1), 138.34 (C2), 127.57-127.14 (C3 and C7), 128.73-128.36 (C4 and C6) and 128.1 (C5). +ve ESI-MS: m/z 391.4 [M + 1] (calc. for [C14H16N2Cl2Pd]=390). UV/Vis in DMSO: λmax [dm3mol^{-}1cm^{-}1)]=275 (2066), 340 (265), 380 (221) nm, in DMSO: water (1:1): λmax [(dm3mol^{-}1cm^{-}1)]=265 (2465), 345(284) nm, in DMSO: phosphate buffer (1:1): λmax [(dm3mol^{-}1cm^{-}1)]=260 (1514), 335 (288) nm.

Biological Evaluation

In vitro anticancer activity

Cell viability was estimated colorimetrically using 2-(3-diethylamino-6 diethylazaniumylidene-xanthen-9-yl)-5-sulfobenzenesulfonate, SRB (sulforhodamine B) as standard assay [25].

Cell lines and culture conditions: Human breast cancer cell lines MCF-7 and MDA-MB-231, were obtained from NCI, USA, and grown in minimal essential medium (MEM). Eagles media were supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma-Aldrich), 2 mM L-glutamine and 1 mM sodium pyruvate (Hyclone) in humidified CO_2 incubator.

Assay of cytotoxicity in cancer cell lines: Cytotoxicity of complex was determined by SRB assay where E5000 cells were seeded into each well of a 96 a well clear flat bottom polystyrene tissue culture plate and incubated for 2 h in MEM. An additional 190 mL cell suspension was added in each well containing 10 mL test sample in 10% DMSO with 10 mL Adriamycin (doxorubicin) as a positive drug control. Each experiment was carried out in 3 replicate wells. After an incubation of 48 h, 100 mL of 0.057% SRB solution (w/v) was added in each well. Then 200 mL of 10 mM Tris base solution (pH 10.5) was added into each well and shaken smoothly. The cell viability was assayed by absorption at 510 nm with a micro plate reader. The experiments were repeated thrice with 3 replicates each time and 99% reproducibility was obtained.

DNA binding

CT-DNA (Sigma) was used as received (analytical grade). Tris– HCl buffer (10 mM, pH=7.2) was prepared in Milli-Q water, for preparation of 50 µM DNA stock solution as well as solutions for absorption titration, viscosity, surface tension, conductivity and zeta potential measurements.

Absorption spectroscopy: DNA concentration was determined using an absorption spectrophotometer as a molar absorptivity (6600 M-1 cm-1) at 260 nm [41,42]. The CT-DNA in buffer gave a ratio of UV absorbance at 260 and 280 nm of 1.8–1.9, indicated the DNA free of protein [43-45]. The complex solution of 10, 30, 50, 70 and 90 µM were prepared in 10% DMSO in Tris buffer, to which the DNA stock solutions (50 µM) were added, (ri=[complex]/[DNA]=0.2, 0.6, 1, 1.4 and 1.8) for absorption titration. The complex+DNA solutions were incubated at r.t. for 15 min. before recording the absorption spectra. To elucidate their binding strength, an intrinsic binding constant (Kb) with CT-DNA was obtained by monitoring a change in absorbance of the DNA with increasing amounts of complex calculated with following equation [46]:

The εa, εf and εb are the apparent, free and bound complex extinction coefficients, respectively. The εf was determined from a calibration curve of an isolated metal complex, with the Beer–Lambert law. The εa was determined as the ratio between the measured absorbance and complex concentration such as Aobs/[Pd] and Aobs/[Pt]. A plot of [DNA]/(εa − εf) vs. [complex] produced a slope of 1/(εb−εf), and a Y intercept equal to 1/Kb(εb − εf); Kb is the ratio of slope to the Y intercept [46].

Physicochemical analysis: Viscosity and surface tension measurements were conducted with BMS at 298.15K controlled temperature with an auto temperature control LAUDA ALPHA RA 8 thermostat [47]. About 15 to 20 measurements for each composition were made for high reproducibility and precision. Flow time and pendent drop were repeated five times to obtain average flow times and numbers of pendent drops. The (η/η0)1/3 vs. binding ratio; the η the dynamic viscosity of DNA with complexes while the η0 is viscosity of DNA mixture in buffer.

Conductance and zeta potentials of DNA solutions with and without complex were measured using LABINDIA, PICO

+conductivity and Microtracz Zetatrac, U2771, DLS, respectively at 25°C. Aqueous KCl at 0.1, 0.01 and 0.001 M of 12.88, 1.413 and 147 mS cm−1respectively were used for calibration of conductivity meter. Likewise, an auto suspended solution of alumina suspension (400-206-100) was used as a zeta potential standard. Initially, for DMF +Tris buffer a set-zero was made for complex. For both the measurements, the DNA concentration was kept constant while the concentration of the complex was varied from 10 to 90 µM with 20 µM intervals.

Scavenging activities

Antioxidant activities have been studied on free radical scavenging of stable 2,2-Diphenyl-1-picrylhydrazyl (DPPH•). Compound solution and DPPH• (0.002%) were prepared in DMF+water (1:1) for complex. For sample preparation, the DPPH• solution was mixed with compound solution in 1:1 ratio, then shaken vigorously and kept in the dark for 30 min. The UV absorbance was measured at 517 nm with a Spectro 2060 plus model UV/Vis spectrophotometer. Radical-scavenging activity was measured as a decrease in absorbance of DPPH•. A lower absorbance of reaction mixture indicated the radical-scavenging activity calculated with the following formula:

The AS is absorbance of DPPH• with a test compound and A0 absorbance of DPPH• without a test compound. Data for antioxidation are presented as means ± SD of three determinations.

Conclusion

The PdBA has not shown an effective anticancer activity against MCF-7 and MDA-MB-231 cell lines but their stronger DBA and antioxidant activity making them suitable for anticancer compounds. Such activity has been correlated with their DBA study supported by DFI. Since, the Pd complexes did not show the anticancer activity but their stronger DBA refer them anticancer compounds, therefore their anticancer investigation on other cancer cell lines could be useful and studies are being under progress. Relative studies of their viscosity and surface tension for DBA have suggested stronger intercalating nature. The zeta potential and conductance data have further revealed stronger DNA interactions as a great support in favour of their SAR. The complexes have also shown significant free radical scavenging activities behaving as antioxidants. Thus, synthesized complex could be used for medicinal purposes. Since, the complex could not show stronger activity on both breast cancer cell line so the anticancer study on other cell lines are also important and it is under process.

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Author Contributions

All experimental work was carried out by Nitin Kumar Sharma. The work was supervised by Prof. Man Singh and Dr. Rakesh Kumar Ameta. The paper was written by Nitin Kumar Sharma.

Conflicts of Interest

The authors declare no conflict of interest.

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