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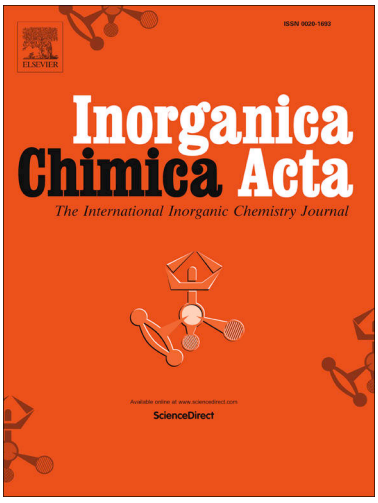

Review article

New Palladium(II) complexes with ONO chelated hydrazone ligand: Synthesis, characterization, DNA/BSA interaction, antioxidant and cytotoxicity

Ganesan Ayyannan, Maruthachalam Mohanraj, Mayakrishnan Gopiraman, Ramasamy Uthayamalar, Gunasekaran Raja, Nanjan Bhuvanesh, Raju Nandhakumar, Chinnasamy Jayabalakrishnan

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New Palladium(II) complexes with ONO chelated hydrazone ligand: Synthesis, characterization, DNA/BSA interaction, antioxidant and cytotoxicity

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Abstract

There is a continuous finding of suitable metal complexes for biomedical and biological applications since the discovery of cisplatin. Two biologically active complexes (**2** and **3**) are synthesized by using 2-chloro-benzoic acid (3,5-dichloro-2-hydroxy-benzylidene)-hydrazide (**1**) ligand. The ligand (**1**) and complexes (**2** & **3**) are characterized by various spectroscopic tools. The molecular structures of (**1**) and (**3**) are confirmed *via*., XRD data. The spectroscopic and XRD data reveals that the ligand coordinates to palladium through ONO chelation and the geometry of the

complexes is distorted square planar. The *in vitro* interaction of the synthesized compounds with calf-thymus DNA (CT-DNA) are analysed through different experiments which shows the intercalation mode of binding. Further, the interaction studies of the compounds with bovine serum albumin protein (BSA) show that, the compounds binds strongly with BSA. Also, antioxidant studies have been carried out with the compounds. Finally, MTT assay reveals that, the Pd(II) complexes displayed significant activity against HeLa and MCF-7 cancer cell lines

Keywords:

Hydrazone; Palladium(II) complexes; crystal structure; DNA/BSA interaction; antioxidant; cytotoxicity.

1. Introduction

Metal complexes have gained considerable development over the past decades in medicinal chemistry. Among the metal complexes, transition-metal complexes are the most widely used chemotherapeutic agents and continue to play a major role in cancer-treatment since the discovery of cisplatin [1-5]. The cisplatin-based compounds have a vast expansion of interest in the use of inorganic compounds for the treatment of tumors [6-8]. Even though cisplatin is most active drug used as a therapeutic agent for a wide range of cancers, it has a number of limitations such as side effects and acquired cell resistance [9, 10]. Therefore, particular effort has been devoted to develop the novel complexes (non-platinum antitumor compounds) that are alternative to cisplatin with higher potency, higher cancer cell selectivity, lower resistance, particularly, that non-platinum complexes bind to DNA under physiological conditions [11].

In recent years, many transition metal complexes were synthesized and evaluated for their antitumor properties [12-16]. Among them, palladium-based complexes have occupied a definite role in chemotherapy since they accumulates in tumors due to specific permeability through cancer cell membranes [17-19]. Moreover, palladium(II) complexes are supposed to be potential anticancer

agents due to close resemblance of Pd complexes with Pt complexes [20]. In addition, the coordination chemistry of the palladium(II) complexes have similar properties with platinum(II) complexes but the higher liability to ligand exchange at the Pd centre (10^5 times greater than Pt) causes rapid hydrolytic processes leading to the dissociation of the complex and the formation of very reactive species, which leads to reduce the biological activity. The above mentioned drawback could be overcome by using bulky hydrazone and chelating ligands. Hydrazone ligand has more chelating capability and structural flexibility that can provide more attention to synthesize metal complexes [21, 22]. Nitrogen and oxygen congaing hydrazone ligands are well biological active compounds [23]. The hydrazone with metal complexes enhances the biological properties than the free hydrazone ligands [24, 25]. In this connection, it is well known that hydrazone type ligand represents a significant class of compounds for chemotherapeutic applications with great potential. However, a very few reports on palladium(II) complexes containing 3,5-dichloro-2-hydroxy-benzaldehyde hydrazone are available in the literature.

In order to explain the antitumor properties of the palladium complexes, better understanding on the binding nature between complexes and DNA is necessary [26, 27], which can be used for the design of new types of antitumor drugs [28-30]. For interaction of metal complexes with DNA, the intercalation mode of binding [31-33] is important because, it influences the anticancer activity of the complexes. In addition, there is interest in the study about interaction between drug-protein interactions that can influence the absorption, distribution, metabolism of amino acids, and excretion potential of metal drugs. The investigation on interaction of proteins with compounds is very useful as it could provide information to determine the therapeutic efficiency and pharmacological response of drugs [34]. Therefore, evaluation of the metal drug molecules towards DNA and BSA are very helpful in order to design and discover the metal-based anticancer therapeutics. Herein, we report the synthesis of hydrazone complexes of palladium by the reaction of $[PdCl_2(EPh_3)_2]$ with H_2L (where E = P or As) in 1:1 metal to ligand stoichiometry ratio. The synthesized ligands and complexes were

characterized by various spectroscopic techniques *via*., UV-Visible, FT-IR and NMR. The crystal structure of ligand and complex **3** was confirmed by X-ray crystallography technique. Finally, the binding properties of compounds with calf thymus (CT-DNA) and bovine serum albumin (BSA) were investigated by different techniques. The radical scavenging and cytotoxicity activities of the synthesized compounds were analysed under *in vitro* conditions.

2. Experimental

2.1. Materials and instrumentation

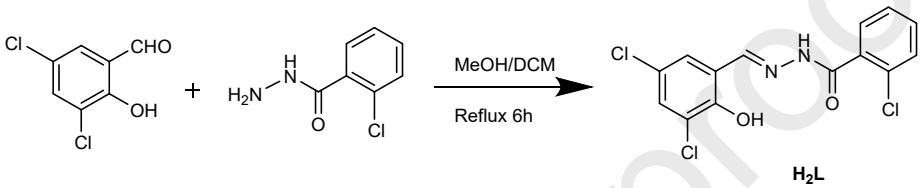
Palladium chloride, triphenyl phosphine, triphenyl arsine, 2-chlorobenzohydrazide and 3,5-dichloro salicyladehyde were purchased from sigma Aldrich. Solvents were used after purification [35]. Bovine serum albumin (BSA) and calf thymus DNA (CT-DNA) (stored at -20 ºC) (Genei) were used as received. Elemental analysis (C, H and N) was carried on a Vario EL III CHNS analyser. Fourier-transform infrared spectroscopy (FT-IR, a Perkin Elmer FT-IR 8000 spectrophotometer) was used to record IR spectra of ligand and complexes. Absorption spectra of compounds were recorded on a Systronics double beam UV-vis spectrophotometer 2202. Emission spectra were recorded on a JASCO FP-8200 spectrophotometer. NMR (^{1}H, ^{13}C and ^{31}P) spectra of the compounds were recorded using Bruker AV III 500 MHZ instrument.

2.2. Synthesis

*2.2.1. Synthesis of hydrazone ligand (**1**)*

Ligand (H_2L) was prepared by refluxing equimolar solutions of 3,5-dichloro-2-hydroxy benzaldehyde (0.1910 g; 1mM) and 2-chlorobenzohydrazide (0.1706 g; 1 mM) in 50 mL of methanol for 6 h. Then the reaction mixture was kept in room temperature until the formation of white colour precipitate. The resultant solid was recrystallized by using methanol/chloroform mixture and the high pure ligand crystal (**1**) was obtained. Yield: 86%; M.P: 178°C. Anal. calcd. for $C_{14}H_9Cl_3N_2O_2$ (%):

C, 48.94; H, 2.64; N, 8.15. Found (%): C, 46.77; H, 2.14; N, 9.6. IR (KBr, cm^{-1}): 3380 ν(OH); 2946 ν(NH); 1656 ν(C=O); 1595 ν(C=N). UV–vis (DMSO), λ_{max} (nm): 298, 362 (π → π*, n→ π*). ^{1}H NMR (500 MHz, DMSO-d_6) δ_H: 12.56 (s, 1H, -NH), 12.21 (s, 1H, -OH), 8.46 (s, 1H, CH=N), 7.69 (d, 1H, *J* = 2.5 Hz, -Ar-H), 7.60 (d, 1H, *J* = 1 Hz, -Ar-H), 7.57 (dd, 1H, *J* = 1.5 & 7.5 Hz, -Ar-H), 7.54 (t, 1H, *J* = 5.0 Hz), 7.48-7.51 (m, 2H, -Ar-H). ^{13}C NMR (125 MHz, DMSO-d_6) δ_C: 168.18 (C=O), 162.46 (C-OH), 152.19 (C=N), 121.19-134.96 (m, -Ar-C).



Scheme 1. Synthesis of ONO hydrazone ligand (**1**)

*2.2.2. Synthesis of palladium(II) complexes [Pd(PPh$_3$)L] (**2**) and [Pd(AsPh$_3$)L] (**3**)*

Initially, the starting compounds, $[PdCl_2(PPh_3)_2]$ and $[PdCl_2(AsPh_3)_2]$, were prepared according to reported literature [36]. In a typical preparation of complex (**2**), two different solutions were prepared; (a) $[PdCl_2(PPh_3)_2]$ (0.7019 g; 1 mM) in dichloromethane (20 mL), and hydrazone ligand (H_2L, 0.3000 g; 1 mM) in methanol (20 mL) (Scheme 2). The above two solutions were stirred with the addition of 2 drops of triethylamine (TEA) and refluxed for 6 h. After reflection, the reaction mixture was concentrated to 5ml and cooled to room temperature. The formed solid was filtered, washed well with methanol and dried under *vacuum*. Similarly, complex **3** was synthesised by using $[PdCl_2(AsPh_3)_2]$ (0.7918 g; 1 mM) and the ligand (**1**) (0.3000 g; 1 mM). Slow evaporation of complexes **2** and **3** from the mixture of methanol/chloroform afforded single-crystals. However, complex **3** is suitable for X-ray diffraction studies whereas complex **2** is not suitable.

$C_{32}H_{22}Cl_3N_2O_2PPd$ ([Pd(PPh$_3$)L], **2**): Yield: 84%, MP: 159 °C, Anal. calcd. (%): C, 54.11; H, 3.12; N, 3.94; Found (%): C, 55.86; H, 4.15; N, 4.72. Selected IR bands (cm^{-1}): 1592 ν(C=N); 1320

ν(enolic C–O); 1426, 1031, 691 (for PPh_3). UV-vis (DMSO), λ_{max}(nm): 297, 361 (intra-ligand transitions); 408, 419 (LMCT). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 8.21 (s, 1H, CH=N), 7.73-7.77 (m, 6H, -Ar-H), 7.64 (dd, 1H, *J* = 1.5 & 7.5 Hz, -Ar-H), 7.49-7.53 (m, 3H, -Ar-H), 7.41-7.45 (m, 6H, -Ar-H), 7.39 (dd, 1H, *J* = 1.0 & 8.0 Hz, -Ar-H), 7.37 (d, 1H, *J* = 2.5 Hz), 7.28-7.31 (m, 2H, -Ar-H), 7.23 (dd, 1H, *J* = 1.0 & 7.5 Hz, -Ar-H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: 174.91 (C=O), 155.91 (C=N), 146.73 (C-OH), 120.71-134.65 (m, -Ar-C). ^{31}P NMR: δ 20.16.

$C_{32}H_{22}Cl_3N_2O_2AsPd$ ([Pd($AsPh_3$)L], **3**): Yield: 78%, MP: 192 °C. Anal. calcd. (%): C, 50.96; H, 2.94; N, 3.71.Found (%): C, 51.42; H, 3.57; N, 3.13. Selected IR bands (cm^{-1}): 1591 ν(C=N); 1323 ν(enolic C–O); 1420, 1095, 687 (for $AsPh_3$). UV-vis (DMSO), λ_{max}(nm): 273, 313, 332 (intra-ligand transitions); 408 (LMCT). ^{1}H NMR (500 MHz, DMSO-d_6) δ_H: 8.21 (s, 1H, CH=N), 7.84 (s, 1H, -Ar-H), 7.69 (d, 8H, *J* = 7.0 Hz, -Ar-H), 7.51-7.64 (m, 7H, -Ar-H), 7.46 (t, 2H, *J* = 7.0 Hz), 7.37 (d, 2H, *J* = 15.5 Hz, -Ar-H), 7.30 (s, 1H, -Ar-H). ^{13}C NMR (125 MHZ, DMSO-d_6) δ_C: 176.42 (C=O), 154.49 (C=N), 147.20 (C=O), 118.13-133.39 (m, -Ar-C).

2.4. Crystal structure determination

A Bruker AXS KAPPA APEX2 CCD diffractometer was performed at room temperature for data collection, crystal screening, and unit cell determination. A graphite-mono chromate Mo Kα radiation (λ = 0.71073 Å) by u and x scans was used for the data collection. The crystallographic data collected were reduced by SAINT program and indexing the reflections and determining the lattice parameters. The SAINT [35] program was adopted for the integration of intensity of reflections and scaling; and the SHELXTL-97 program was ran for space group and structure determination, and full-matrix least-squares refinements on F^2. All non-hydrogen atoms were refined anisotropically, while the hydrogen atoms were placed in calculated by using the HFIX instruction.

2.5. Stability test

Complexes **2** and **3** were first dissolved in DMSO (0.5% of the final volume) and then diluted with tris-HCl buffer (99.5% of the final volume) to a required concentration. The hydrolysis profiles of these complexes were recorded by monitoring the electronic spectra of the resulting mixture over 24 h.

2.6. Biological Evaluation

2.6.1. DNA binding studies

The interaction of synthesized compounds with DNA was studied by using absorption spectroscopy. In fact, it is very useful technique to understand the mode of binding between the drug molecule and DNA and to calculate the binding constant value (K_b). Initially stock solution of CT-DNA was prepared in tris(hydroxymethyl)amino methane (Tris, 5mM) and NaCl (50 mM), and the P^H was adjusted to 7.2 with HCl. The stock solutions of compounds were prepared in 5% of dimethyl sulfoxide (DMSO) and diluted with 95% of tris-HCL buffer solution to the required concentration. The absorption experiment was carried out by keeping the concentration of the synthesized compounds constant (25μM) and varying the concentration of DNA (0-50μM). The absorption was recorded for each addition [36]. The binding constant (K_b) was calculated using Wolfe-Shimer equation.

The competitive ethidium bromide studies were performed in the absence and presence of synthesized compounds. Addition of test compound solutions to EtBr-DNA resulted in the decrease of the emission intensity. From the emission intensity values, the quenching constant value was calculated using the following equation,

$$F_0/F = K_q\,[Q] + 1$$

Electrochemical technique was used to explore the interaction between DNA and synthesized compounds and to verify the interaction mode. In this experiment the addition of DNA to complex

solution and the electrical potential of the complex were recorded. In addition, the viscosity of synthesized compounds was carried out in absence (η_0) and presence (η) of compounds. The binding properties of the compounds were found out using $\eta = (t\text{-}t_0)/t_0$, where t and t_0 are the obtained flow time for the compounds.

2.6.2. BSA binding studies

The binding ability of synthesized ligand and complexes with BSA protein was determined by using absorption and fluorescence spectroscopic techniques. The BSA stock solution (1μM) was prepared with tris-HCl buffer (pH=7.2) and it was stored in a dark room for further analysis. The fluorescence quenching spectra were recorded at room temperature with constant concentration of protein while varying the concentration of compounds (**1**-**3**). Further, the BSA interaction with compounds was studied using absorption spectra. The concentration of compounds and BSA protein was from 0-25 μM and 1μM, respectively.

2.6.3. Antioxidant assays

The radical (DPPH, NO and OH) scavenging activity of the synthesized compounds was studied by using previously reported methods [37-39]. The experiments were carried out in triplicate by varying the concentration of compounds (10-50 μM). The activity percentage was calculated by using the relation, % activity = $[A_0\text{-}A_c] \times 100$, where A_0 and A_c represent the absorbance in the absence and presence of compounds. In addition, ascorbic acid and methanol was used for positive and negative controls, respectively. From the percentage of activity, the 50% of antioxidant potential (IC_{50}) value was calculated.

2.6.4. MTT assay

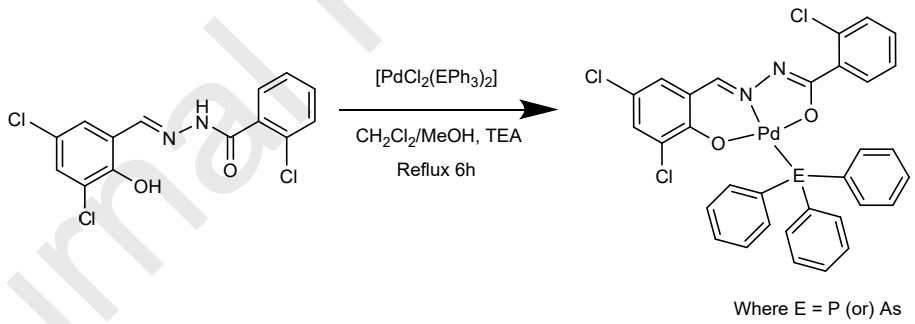
In vitro cytotoxicity of synthesized compounds was analyzed on human cervical (HeLa) and human breast cancer cell (MCF-7) [40]. For this assay, the test compounds were dissolved in DMSO and blank samples containing same volume of DMSO were taken as controls to identify the activity

of solvent in experiments. These solutions were incubated for 4 hours. Percentage of inhibition was calculated by using following equation, % cell inhibition = 100-Abs (sample)/Abs(control) × 100. The IC_{50} values were calculated from the cell viability curve of the graph plotted between % of inhibition and compound concentration.

3. Results and discussion

3.1. Synthesis and spectroscopic investigation

Palladium(II) complexes were synthesized by refluxing the methanolic mixture of 2-chloro-benzoic acid (3,5-dichloro-2-hydroxy-benzylidene)-hydrazide, dichloromethane solutions of palladium precursors and triethylamine for 6 h. It was confirmed that the obtained compounds are air stable and have good solubility in DMF, DMSO, methanol and 1% DMSO/50 mM Tris-HCl buffer solution. The molecular structure of the ligand (**1**) and complexes (**2** and **3**) was determined by elemental analysis and various techniques, *viz*. FT-IR, UV-Visible, ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectroscopy. The molecular geometry of **1** and **3** was confirmed by the XRD study.



Scheme 2. Synthesis of complexes

The spectroscopic data (FT-IR, UV-visible, ^{1}H and ^{13}C NMR) were collected for ligand and complexes (**2** and **3**) and it was used to determine the mode of coordination of the hydrazone ligand to palladium ion in complexes. The ligand showed strong bands at 2946 (N-H) and 1656 cm^{-1} (C=O) whereas these bands disappeared in FT-IR spectra of the complexes. Similarly, free hydrazone displays band at 1595 cm^{-1} corresponding to the azomethaine (C=N), whereas, in the case of

complexes, the peak shifted towards lower values [1592 (**2**) and 1591 cm^{-1} (**3**)]. The NH peak disappeared completely in complexes (former), indicating the involvement of enol form of oxygen in coordination with the palladium(II) ion [41]. The later proves the coordination of C=N group to the palladium centre [42]. Complexes have characteristic vibrations appeared for triphenyl phosphine and triphenyl arsine in expected region. The electronic spectra of ligand and complexes showed two to four bands in the region 293-419 nm. The bands of complexes (**2** and **3**) at 297-273 nm and 361-332 nm are due to the π-π* and n-π* intra ligand transition, respectively. The enolization followed by the deprotonation of the ligand during complexation was confirmed by observing a shift in the n-π* transition which appears in the region of 298 nm. Absorption spectra of complexes showed broad band with less intensity (408 and 419 nm), which can be assigned to the ligand to metal charge transfer transition (LMCT). Broadness of the bands may be due to the overlapping of the LMCT transitions of O → Pd and N → Pd [43, 44].

In the ^{1}H NMR spectrum of ligand H_2L, the chemical shift for the hydroxyl proton (-OH) appears at 12.21 ppm, whereas, the complexes show no signal in the region. Alike, a weak singlet appears at 12.56 ppm (due to NH proton), whereas, no such resonance was attributed to NH proton signal for complexes **2** and **3**. These observations reveal that phenolic oxygen is involved in the complexation with Pd(II) ion and also the enolate oxygen atom coordinated to the metal centre after enolisation followed by deprotonation [45]. Further, the singlet at 8.46 ppm confirmed the presence of azomethine proton (-CH=N). In case of complexes **2** and **3** the singlet at 8.45 ppm was shifted to 8.21 ppm in both the complexes due to the coordination of azomethine N with Pd(II) ion [46]. The region of 7.20-7.83 ppm is ascribed to the aromatic protons for both in ligand and complexes. The ^{13}C NMR spectrum of ligand H_2L showed the chemical shift of carbonyl carbon (C=O) at 168 ppm which is completely disappeared for complexes. Whereas new signals were noticed for the Pd-complexes at 155 and 154 ppm (C-O), indicates the coordination of oxygen *via* deprotonation [47]. The ligand displayed a single resonance at 162 ppm due to the azomethine carbon atom [48]. The

downfield shift of this signal for the complexes at 174 and 176 ppm clearly indicates the coordination of the azomethaine group. Signals in the regions of 118-152 ppm confirmed the aromatic carbons of the ligand and complexes. Further ^{31}P NMR spectrum was recorded for the complex **2**. A sharp singlet was observed at 20.8 ppm, thus suggested the presence of triphenylphosphine group in complex **2** [49].

Overall, the spectroscopic results confirmed the coordination mode of benzoylhydrazone ligand to palladium (II) ion *via* the phenolate oxygen (O), the azomethine nitrogen (N) and imidolate oxygen (O) and also the presence of PPh_3/$AsPh_3$ groups.

3.2. Crystal structure description of H_2L (1) and complex (3)

The single crystal X-ray diffraction studies confirmed the structure of the ligand **1** and the molecular structure of the ligand is shown in Fig. 1. The selected bond distances and angles are listed in Table 2. The ligand crystallizes in monoclinic C2/*c* space group and eight molecules present in a unit cell. The successful formation of hydrazone ligand is confirmed by using the hydrazone C(8)-N(2) bond length, 1.278(2) Å which confirmed the formation of azomethine C=N bond. The presence of hydrazinic (N-H) hydrogen and bond distances (C(7)-O(1), 1.229(2) Å) in the ligand confirms that the hydrazone exists in keto form. The N(1)-N(2) (1.3713(19) Å) and N(1)-C(7) (1.346(2) Å) bond distances in H_2L are in between the ideal values corresponding single bonds [N-N, 1.45 A; C-N, 1.47 A] and double bonds [N=N, 1.25 A, C=N, 1.28 Å], as obvious for an extended π-delocalization throughout the ligand molecule. The hydrazine nitrogen N(1) and the oxygen O(1) atoms are presented in a *tans* manner with corresponding to C(7)-N(1); with the bond length of 1.346(2) Å.

ORTEP diagram (Fig. 2) shows ONO pincer type of ligand coordinated with central metal palladium ion *via* the phenolate oxygen, azomethine nitrogen and deprotonated imidole oxygen, each forming five membered and six membered chelate rings. Complex (**3**) was crystallized in C_2/c space

group in the *monoclinic* crystal system. Selected refinement data are represented in table 1. ORTEP representation of the complex (**3**), is shown in Fig. 2 and unit cell packing diagram of the complex **3** is provided in ESI. In complex **3** bond lengths are 1.971(3) Å Pd(1)-N(1), 1.971(2) Å Pd(1)-O(1) and 1.993(2) Å Pd(1)-O(2) and 2.4121(4) Å Pd(1)-As(1). The *trans* angles of the complexes O(1)-Pd(1)-O(2) and N(1)-Pd(1)-As(2) are 174.60(10) and 177.68(8) respectively. The selected bond lengths and bond angles confirmed the square planar geometry around the central metal palladium with significant distortion. Bond length and bond angle of complex **3** are in well agreement with the previous data of palladium(II) complexes [50]. The spectroscopic and single crystal XRD studies strongly suggested that the hydrazone ligand is coordinated to the Pd ion through ONO in a tridentate fashion.

3.3. Stability Assays

Stability of synthesized compounds was investigated in DMSO Tris-HCl buffer as a solvent. In fact, the DMSO is the most common solvent used to prepare stock solutions for many biological testing and it can act as a ligand in some transition metal complexes (can coordinate the metal centre *via* either the S or O atom). The time-dependent UV-vis spectral scans found that the present ligand is stable in aqueous DMSO even after extended time intervals, indicating its suitability for cellular studies in an aqueous medium. The UV-visible absorption spectra of the synthesized complexes **2** and **3** were recorded in buffer-DMSO solution (Fig S6†). From absorption spectra no changes (either in both intensity and position of bands) were observed after a 24 h of period, which strongly indicate the stability of the complexes in buffer-DMSO solution.

3.4. Nucleic acid binding studies

UV-Vis spectrophotometry, fluorescence, electrochemical, and relative viscosity measurement were used to confirm the interactions of the synthesized compounds with CT-DNA.

The binding ability of the compounds with CT-DNA was investigated by absorption titration technique. Fig. 3 shows the absorption spectra of the ligand and complexes in absence and presence of CT-DNA. Increasing the amount of CT-DNA to the ligand (**1**) the spectrum exhibited a hypochromism of about 31% (at 292 nm) and 37% (357 nm) with 4 nm red shifts. Interestingly, complex **2** exhibited a hypochromism of about 58%, 56% and 57% with a red shift of 7, 4 and 1 nm at 264, 315 and 420 nm and absorption bands of **3** at 267, 339 and 319 nm exhibited the same incident of hypochromism of about 31%, 30% and 29% respectively with a red shift of about 1, 5, 1 nm. From the absorption changes of parent ligand and complexes, the binding to CT-DNA was confirmed [51, 52]. In comparison to ligand, the complexes showed better hypochromicity with red shift. This phenomenon is obviously due to the better ability of Pd(II) complexes (**2** and **3**) than the parent ligand. Binding properties were evaluated through the binding constant K_b which can be obtained from the following equation,

$$[DNA]/[\varepsilon_a-\varepsilon_f] = [DNA]/[\varepsilon_b-\varepsilon_f] + 1/K_b[\varepsilon_b-\varepsilon_f]$$

where [DNA] is the concentration of DNA in base pairs, ε_a, ε_b and ε_f are the extinction coefficient of apparent, fully bound and free compounds in solution respectively. From the absorption values plot of $[DNA]/[\varepsilon_a-\varepsilon_f]$ *versus* [DNA] assigned as a $1/[\varepsilon_b-\varepsilon_f]$ while y-intercept is $1/K_b[\varepsilon_b-\varepsilon_f]$. The binding constant K_b is the ratio of slope/ y-intercept (Fig. S7†). The intrinsic binding constant (K_b) values are 1.3×10^4 M^{-1} (ligand **1**), 7.1×10^4 M^{-1} (**2**) and 6.3×10^4 M^{-1} (**3**). From the binding constant values shows intercalation mode of binding between the CT-DNA and compounds. The K_b values of the present Pd-complexes are confirm that bind to DNA helix *via* intercalative mode [53].

Further ethidium bromide (EtBr) fluorescence quenching experiment was carried out for compounds (**1-3**) and the data is shown in Fig.4. The addition of compounds to EtBr causes a significant amount of decrease in emission intensities, indicating that the compounds competitively

bound to CT-DNA with EtBr. Further, the emission intensity values were utilized according to the Stern-Volmer equation.

$$F_0/F = K_q [Q] + 1$$

where F_0 and F is the emission intensity of absence and presence of compounds, K_q is the quenching constant and [Q] is the concentration of the compound. The K_q Value is obtained as a slope from the plot of F_0/F *versus* [Q] (Fig. S8†). The K_q values for compounds were 1.58 x 10^4 M^{-1}, 4.75 x 10^4 M^{-1} and 3.03 x 10^4 M^{-1} for the compounds (**1-3**) respectively. In addition apparent binding values (K_{app}) are calculated by following equation.

$$K_{EB}[EB] = K_{app} [compound]$$

where the DNA binding constant of EB, [EB] is the concentration of EB=10μM and they were (1.1 × 10^5 M^{-1}), (3.5 ×10^7 M^{-1}) and (2.2 ×$10^7 M^{-1}$) for compound **1**, **2** and **3** respectively. The above the results show that complex **2** has more ability to bind with DNA. The binding and apparent constant values express the interaction between DNA and complexes should be intercalation [54].

Electrochemical techniques have been used to study the binding properties of complexes with DNA in order to verify the DNA modes suggested by the spectroscopic studies. DNA is added to complexes, the new redox peak did not appear while intensity of current decreases, this changes reveals the interaction between DNA and complexes are confirmed (Fig.5). The current intensity is decreased in both the complexes due to the electrode surface bound with equilibrium mixture of free DNA and palladium complex. Moreover addition of DNA showed a positive shift at the cathodic (ΔE_{pc}) potential and anodic (ΔE_{pa}) potential of the complexes which evidences the presence of intercalative binding properties [55]. The electrochemical studies are well acknowledged with absorption and fluorescence studies.

Viscosity studies were useful to understand about binding properties of the ligand and complex to DNA. Viscosity of DNA depends on both the size and shape of DNA. Viscosity of DNA increases due to an increase in the DNA helix lengths. In fig. 6 shown that presence and absence of compounds (**1**-**3**) in DNA solution. The relative viscosity of DNA bind to ligand and complexes increases with increases in concentration. Increase in degree of viscosity indicates the intercalative mode of binding of the compounds in the order of **2**>**3**>**1**. Therefore, above DNA binding studies confirmed that the metal complexes have more binding affinity than free ligand due to complexes has more planar properties than ligand.

3.5. BSA binding studies

3.5.1. Fluorescence quenching mechanism

Protein binding properties of the ligand and complexes (**1**-**3**) with BSA protein studies were carried out [56]. Change in fluorescence spectra in absence and presence of quencher provides the information about nature of the binding properties. Fluorescence binding experiment was studied by increasing the concentration of H_2L and palladium(II) complexes (0-25μM) to BSA (1μM) at room temperature. Experiments exhibits peak at 346 nm due to tryptophan residue it excited at 296 nm were measured using the compounds as quenchers with increasing concentrations (Fig. 7). Upon addition of test compound solution to BSA, the fluorescence intensity of tryptophan decreased significantly which confirm the interaction between test compounds and BSA. Fluorescence intensity of tryptophan moiety decrease in order to 65%, 72%, and 70% were obtained for compound **1**, **2** and **3** respectively. Emission intensity changes would provide the interaction of compounds with BSA. It could cause conformational changes in BSA fluorophore tryptophan environment [57]. Further fluorescence quenching is expressed by the Stern-Volmer relation,

$$F_0/F = 1 + K_q\,[Q]$$

where F_0 and F are the absence and presence of quencher fluorescence intensities, K_q is quenching constant and [Q] quencher concentration. K_q value of the test compounds calculated from the plot of F_0/F *versus* [Q] (Fig. S9†) in respect to ligand and complexes **1**, **2** and **3** were found to be 5.4×10^4 M^{-1}, 1.2×10^5 M^{-1} and 7.3×10^4 M^{-1}, respectively. The quenching constant value strongly suggests the interaction of the compounds with BSA biomolecule [58, 59].

3.5.2 Binding constant and binding sites

Further, the binding constant and number of binding sites can be calculated by using following Scatchard equation,

$$\log [(F_0\text{-}F)/\mathrm{F}] = \log K_{bin} + n \log[\mathrm{Q}]$$

where K_{bin} is the binding constant of synthesized compounds with BSA and n is the number of binding sites. The binding constant (K_{bin}) and the binding sites (n) have been obtained from the plot of $\log[F_0\text{-}F]$ *versus* log[Q] (Fig. S10†). From slope values, the binding constant K_{bin} are calculated, 5.9×10^4 M^{-1} (**1**), 7.7×10^5 M^{-1} (**2**), 7.1×10^4 M^{-1} (**3**) and binding sites are 0.92 (**1**), 1.45 (**2**) and 1.21 (**3**) respectively. The binding constant (K_{bin}) values suggested that the synthesized complexes have more binding affinity than ligand. The obtained binding site value n (~1) strongly supported the existence of single binding site in BSA protein. Calculated K_q and K_{bin} values are proved that complex **2** has more interaction with BSA due to presence of triphenylphosphine as a co-ligand and thus showed a higher binding activity.

3.5.3. UV-Vis absorption

Absorption spectroscopic technique is very simple and easy way for exploring the type of quenching. Generally, BSA solution showed a strong band around 200 nm due to protein backbone and another one band shown around 278 nm that is related to absorption of aromatic amino acids (Trp, Tyr, and Phe). The absorption spectrum of the BSA solution and BSA-compounds were shown

in Fig. 8. From the spectra, the intensity range of BSA was enhanced and small blue shifts were observed about 1, 3 and 2 nm for **1**, **2** and **3** respectively. The results revealed the existence of a static quenching mechanism in the complexes formation between BSA and palladium(II) complexes [60].

3.6. Antioxidant activities

The antioxidant potentials (as IC_{50} values) of the present test compounds against reactive radical species (DPPH, OH and NO) are given in table 3 (Fig. S11†). The IC_{50} value shows that the synthesized ligand and palladium(II) complexes exhibit antioxidant properties in the order of **2**>**3**>**1** in all three radicals. In comparison to the free ligand, the Pd-complexes possess higher antioxidant activity. This is due to chelation of the hydrazone ligand with the Pd(II) ion and more planarity of the complexes [61-63]. Present significant aspect of the investigation is complex **2** produced excellent antioxidant activities that are comparable with those of standard antioxidant ascorbic acid (Ac).

3.7. *In vitro* cytotoxicity MTT Assay

The *in vitro* cytotoxicity results of ligand and complexes are summarized in table 4. From the results, ligand has poor cytotoxicity value in all the tested tumour cell lines. The palladium(II) complexes (**2** and **3**) exhibited significant cytotoxic effect in lower concentration. In particular, complex **2** have better activity than other. The good cytotoxicity activity of new complexes are attributed to the extended planar structure induced by the $\pi \rightarrow \pi^*$ conjugation resulting from the chelation of the ligand to complexes [64, 65]. Furthermore, the high lipophilic character owing to the presence of triphenyl phosphine group may also be the reason for the better cytotoxicity of complex **2.** Moreover, comparison of the results inferred that the present palladium(II) complexes have more activity than reported results [66].

4. CONCLUSION

Two square planar Pd(II) complexes were successfully synthesized from 2-chloro-benzoic acid (3,5-dichloro-2-hydroxy-benzylidene)-hydrazide ligand and characterized. Their biological properties were also investigated. From UV−vis spectroscopic titration, EtBr displacement assay, electrochemical and viscosity studies, it is confirmed that the ligand and complexes bind with CT-DNA through the intercalation mode due to higher planar aromatic rings and $\pi \rightarrow \pi^*$ stacking interactions, which initiate the intercalation in to the base pair of DNA double helices. Absorption and fluorescence emission study indicates that the complexes have good binding properties with BSA. The ligand and the complexes (**2** and **3**) have been screened for their *in vitro* free radical scavenging activity. The complexes exhibit more ability to scavenge DPPH, OH and NO radicals and significantly high scavenging activity was observed against superoxide and hydroxyl radicals. Complex **2** is the most active radical scavenger than the other. The *in vitro* cytotoxicity of the ligand and complexes on tumour cell lines (HeLa and MCF-7) have been assessed by MTT and the results indicates that complex **2** shows better antitumor activity than ligand **1** and complex **3**. Hence, the complexes **2** and **3** display significant biological properties compared to the previous reports and may be helpful to understand the mechanism of complexes with biomolecules, and also useful in the development of new potential anticancer agents. In particular, the complex containing triphenylphosphine coligand was found to be extremely effective.

Supporting information:

Electronic supplementary information (ESI) available: Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication numbers (CCDC 1504793 and CCDC 1504704, for **1** and **3** respectively). e-mail: deposit@ccdc.cam.ac.uk ; Web site http://www.ccdc.cam.ac.uk).

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Manuscript Title:

New Palladium(II) complexes with ONO chelated hydrazone ligand: Efficient candidates for DNA/BSA interaction, antioxidant and cytotoxicity

All the authors declared no conflict of interest

Highlights

- Two palladium(II) hydrazone complexes were synthesized and characterized.

- Molecular structure of ligand (**1**) and complex (**3**) was confirmed by X-ray diffraction study.
- The complexes exhibited intercalation binding ability on DNA/BSA.
- The synthesized complexes showed good antioxidant activities.
- The cytotoxicity *in vitro* of synthesized compounds against two tumor cell lines was investigated.

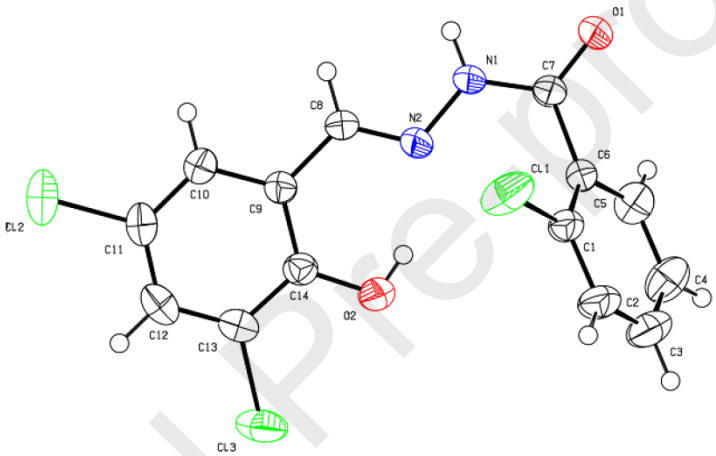


Fig. 1. ORTEP diagram of **1** with thermal ellipsoid at 50% probability

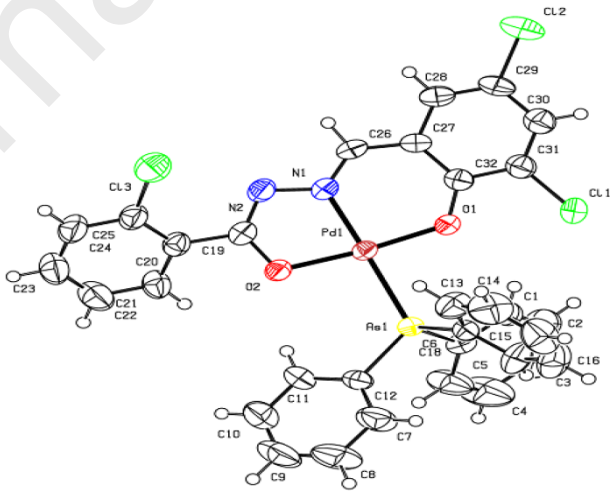


Fig. 2. ORTEP diagram of **3** with thermal ellipsoid at 50% probability

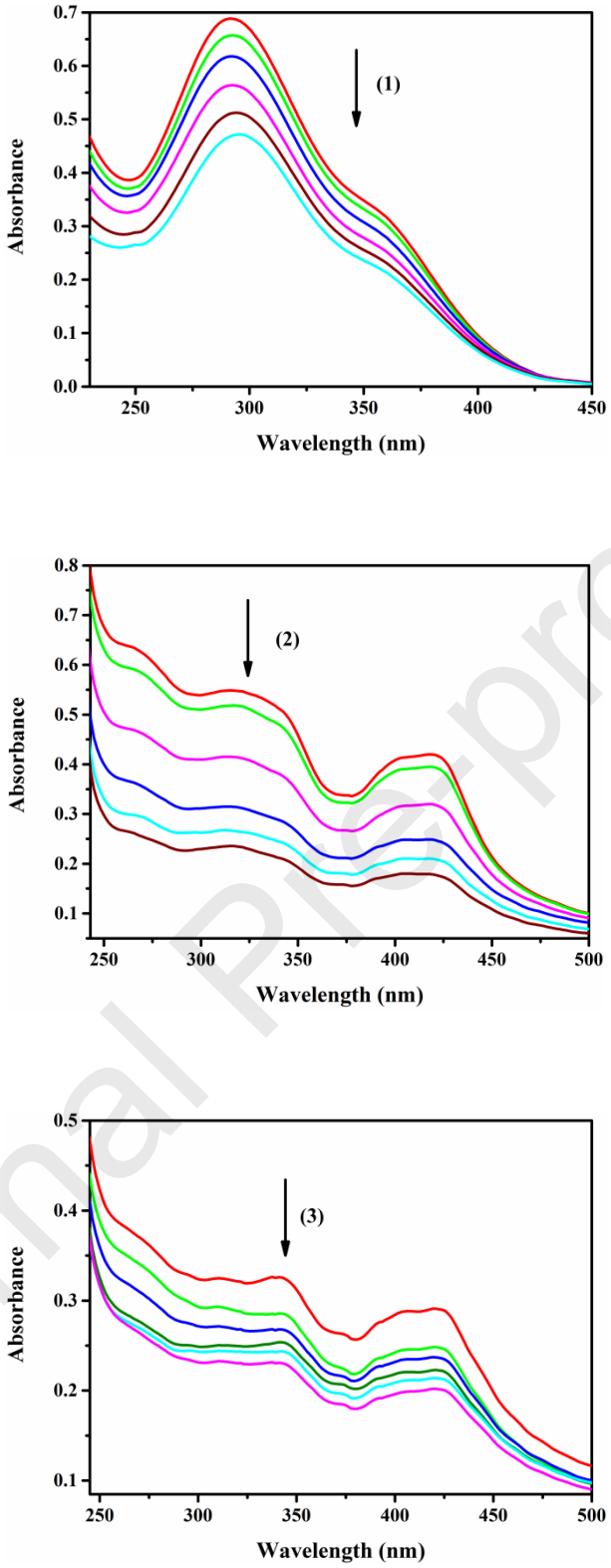


Fig. 3. Electronic spectra of the compounds (**1**), (**2**) and (**3**) in Tris-HCl buffer upon addition of CT-DNA. [Compound] = 25μM, [DNA] = 0-50μM. Arrow shows that the absorption intensities decrease upon increasing DNA concentration

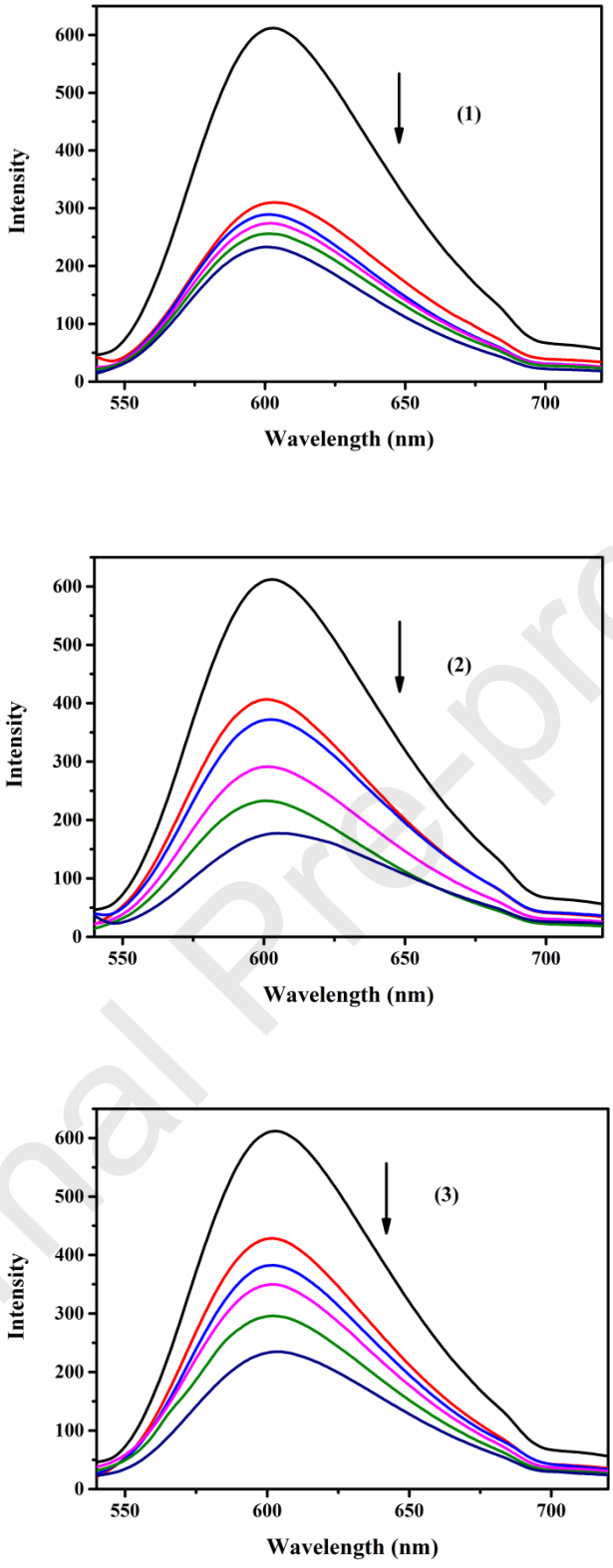
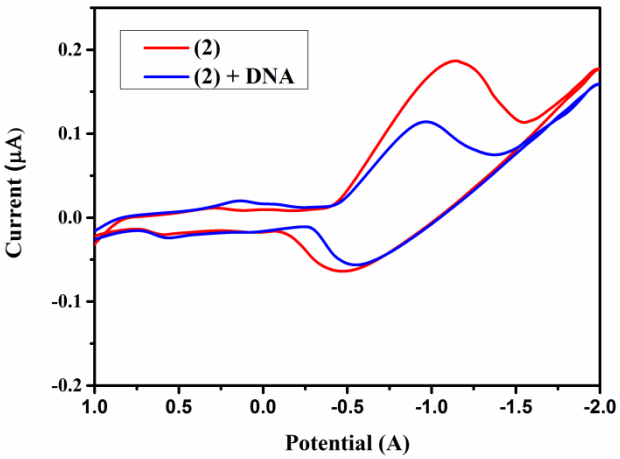


Fig. 4. Fluorescence quenching curves of ethidium bromide bound to DNA: 1, 2 and 3. [DNA] = 10μM, [EB] = 10μM, and [complex] = 0-50 μM. Arrow shows the emission intensity changes upon increasing complex concentration.



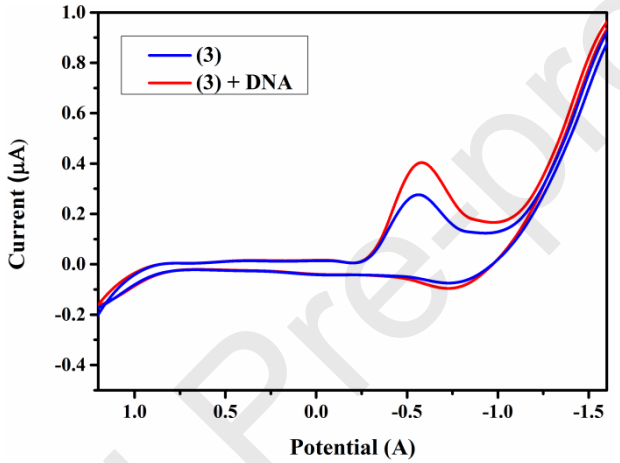


Fig. 5. Cyclic voltammogram of complex **2** and **3** in the absence and presence (inner line) of DNA (10 μM). Scan rate: 100 mV s^{-1}

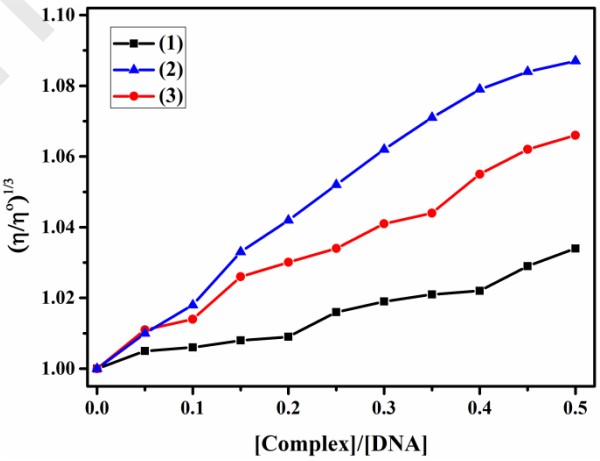


Fig. 6. Effect of the compounds (**1-3**) on the viscosity of CT-DNA.

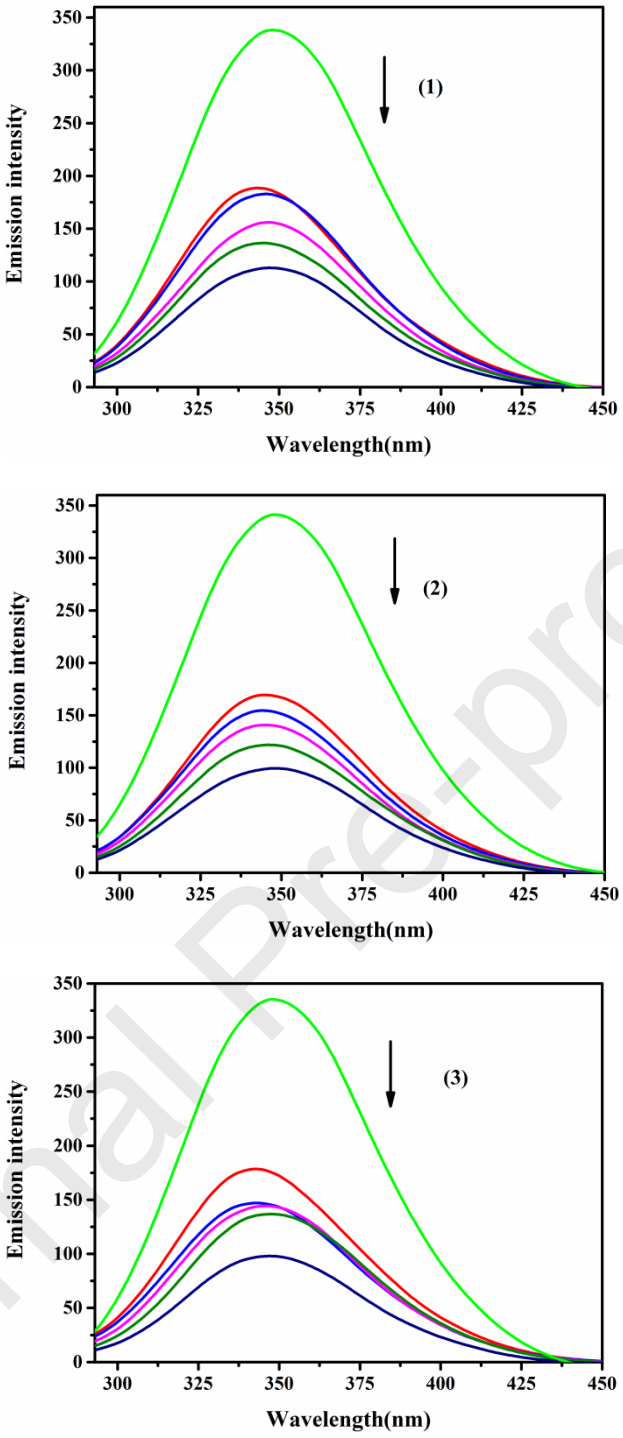


Fig. 7. Fluorescence titrations of **1-3** (0-25μM) with BSA (1μM).

Compound	**1**	**3**
Empirical formula	C_{14} H_9 Cl_3 N_2 O_2	C_{32} H_{22} As C_{l3} N_2 O_2 Pd
Formula weight	343.58	754.19
Temperature	296(2) K	296(2) K
Wavelength	0.71073 Å	0.71073
Crystal system	monoclinic	monoclinic
Space group	C2/*c*	C2/*c*
Unit cell dimensions		
a (Å)	26.612(3)	27.5155(13)
b (Å)	6.4992(6)	9.1058(4)
c (Å)	18.059(2)	26.9787(11)
α ( )	90	90
β ( )	111.626(6)	115.946(4)
γ ( )	90	90
Volume (Å^3)	2903.6(5)	6078.2(5)
Z	8	8

Table 1.

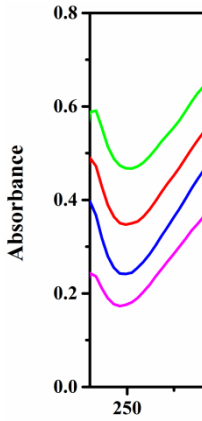


Fig. 8. Absorbance titrations of **1-3** with BSA.

Crystal and structure refinement data.

Density (calculated) Mg/m^3	1.572	1.648
Absorption coefficient mm^{-1}	0.635	1.987
F(000)	1392	1588.9
Crystal size/mm^3	0.50 x 0.35 x 0.30	0.40 x 0.30 x 0.30 mm
Theta range for data collection (°)	2.43 to 28.26°	1.65 to 28.32 °
Index ranges	-35<=h<=34, -8<=k<=8, -24<=l<=24	-36<=h<=36 , -12<=k<=12 , -22<=l<=35
Reflections collected	10809	24517
Independent reflections	3581 [R(int) = 0.0189]	7471 [R(int) = 0.0358]
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	3581 / 0 / 193	7471 / 0 / 370
Goodness-of-fit on F^2	1.021	1.126
Final R indices [I>2σ(I)]	R_1 = 0.0390, wR_2 = 0.1030	R_1 = 0.0382 , wR_2 = 0.0938
R indices (all data)	R_1 = 0.0501, wR_2 = 0.1122	R_1 = 0.0682 , wR_2 = 0.1196
Largest diff. peak and hole e.Å^{-3}	0.280 and -0.414	0.657 and -1.048

Table 2. Selected bond lengths [Å] and angles [°] of ligand (**1**) and palladium(II) complex **3**.

Bond lengths	**1**	**3**	
N(2)-C(8)	1.278(2)	N(1)-Pd(1)	1.971(3)
N(1)-N(2)	1.3713(19)	O(1)-Pd(1)	1.971(2)
N(1)-C(7)	1.346(2)	O(2)-Pd(1)	1.993(2)

O(1)-C(7)	1.229(2)	As(1)-Pd(1)	2.4121(4)
O(2)-C(14)	1.344(2)	N(1)-N(2)	1.395(4)
Cl(1)-C(1)	1.7323(18)	C(19)-O(2)	1.317(4)
Cl(2)-C(11)	1.7357(18)	C(26)-N(1)	1.284(5)
Cl(3)-C(13)	1.7294(18)	C(32)-O(1)	1.314(4)
Bond angles			
C(7)-N(1)-N(2)	121.76(13)	O(1)-Pd(1)-O(2)	174.60(10)
C(8)-N(2)-N(1)	116.17(13)	N(1)-Pd(1)-As(1)	177.68(8)
O(1)-C(7)-N(1)	119.78(14)	N(1)-Pd(1)-O(2)	80.14(11)
O(2)-C(14)-C(9)	122.84(15)	O(1)-Pd(1)-N(1)	94.51(11)

Table.3. Antioxidant activity of synthesized compounds and standard ascorbic acid.

Compounds	IC_{50} (μM)		
	$DPPH^{\cdot}$	$OH^{\cdot}$	$NO^{\cdot}$
1	95.11	90.73	86.17
2	38.32	10.64	47.91
3	44.76	14.71	53.03
Ascorbic acid (Asc)	27.71	5.12	37.52

Table 4. *In vitro* Cytotoxic activity of the ligand and palladium(II) complexes against the human cervical (HeLa) and human breast (MCF-7) cancer cell lines.

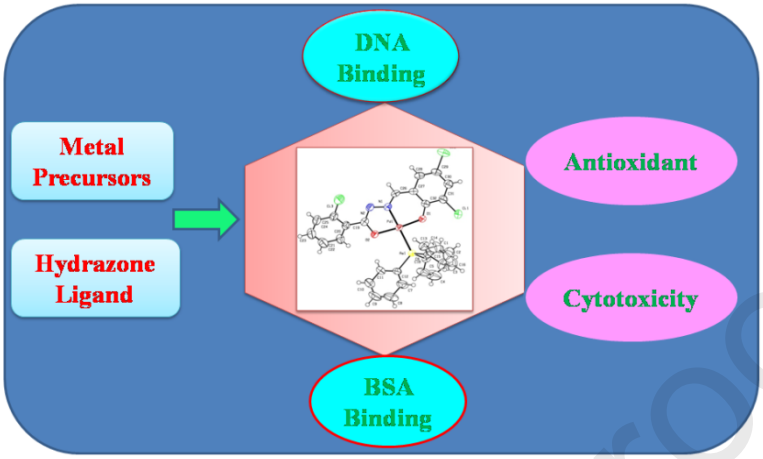
Compounds	IC_{50} (μM)	
	HeLa	MCF-7
1	85.54	93.71
2	23.93	26.49
3	32.65	36.42
Cisplatin	16.21	15.35

New Palladium(II) complexes with ONO chelated hydrazone ligand: Synthesis, characterization, DNA/BSA interaction, antioxidant and cytotoxicity

Graphical abstract

New palladium(II) complexes containing 3,5-dichloro-2-hydroxy-benzaldehyde hydrazone were synthesized and characterized by various analytical, spectral and crystallographic techniques. The synthesized compounds have been subjected to study their DNA and protein binding affinity

with CT-DNA and BSA protein. And their antioxidant activity and *in vitro* cytotoxic potential were evaluated.



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