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Synthesis, characterization and biological activity of trans-platinum(II) complexes with chloroquine

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Abstract

Three platinum-chloroquine complexes, *trans*-Pt(CQDP)₂(I)₂ [**1**], *trans*-Pt(CQDP)₂(Cl)₂ [**2**] and *trans*-Pt(CQ)₂(Cl)₂ [**3**], were prepared and their most probable structure was established through a combination of spectroscopic analysis and density functional theory (DFT) calculations. Their interaction with DNA was studied and their activity against 6 tumor cell lines was evaluated. Compounds **1** and **2** interact with DNA primarily through electrostatic contacts and hydrogen bonding, with a minor contribution of a covalent interaction, while compound **3** binds to DNA predominantly in a covalent fashion, with weaker secondary electrostatic interactions and possibly hydrogen bonding, this complex also exerted greater cytotoxic activity against the tumor cell lines.

Keywords

Cancer; Chloroquine; Platinum; DNA; Anticancer activity

1. Introduction

The development of modern medicinal chemistry was stimulated by the discovery of cis-diamminedichloro platinum(II) (cisplatin) [1,2], one of the most widely used drugs for the treatment of cancer, particularly genitourinary, and head and neck cancers [3]. Through an understanding of its chemistry and mechanisms of action, many analogues have been synthesized with the aim of enhancing the therapeutic activity and circumventing intrinsic or acquired drug resistance [4].

The *trans* analogue, trans-diamminedichloro platinum(II) (transplatin) shows no anticancer activity and, as many other complexes in the *trans* configuration also were found to be ineffective, it was assumed that a *cis* configuration of the labile groups was required for the

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antitumor activity of such compounds. The lack of biological activity of transplatin is due to its kinetic instability and consequent susceptibility to deactivation. However, more recently, some trans platinum(II) complexes have shown good antitumor activity *in vitro* and *in vivo* [5,6,7]. The replacement of one or both amines ligands in transplatin with more bulky ligands, may retard the substitution reaction of chloride ions, and thus reduce undesirable reactions between the platinum center and other biomolecules present in plasma which inhibit its interaction with DNA.

Chloroquine diphosphate (CQDP, Fig. 1), an antimalarial lysosomotropic base, is known for its anti-inflammatory effects and is therefore also used for the treatment of autoimmune diseases [8,9]. Interestingly, chloroquine (CQ) has been shown to display some anticancer activity [10,11] as well as a protective effect [12,13,14,15,16,17]. Previous studies have demonstrated that the coordination of chloroquine (CQ) to metal-containing fragments such as Pt [18], Pd [19] and Ru [20, 21] leads to interesting anticancer activity.

Based on these observations, we have undertaken the synthesis and characterization of three new Pt-CQDP and Pt-CQ derivatives, Pt(CQDP)₂(I)₂ [1], Pt(CQDP)₂(Cl)₂ [2], and Pt(CQ)₂(Cl)₂ [3], and the study of their interaction with DNA. Additionally, their cytotoxicity against 6 tumor cell lines was evaluated.

2. Experimental Section

2.1. General

All manipulations were routinely carried out under N₂ using common Schlenk techniques. Solvents were purified by standard procedures immediately prior to use. CQDP, calf thymus DNA (CT-DNA), buffers and solvents were purchased from Sigma-Aldrich Co. The extraction of the CQ base has been described previously [22]. All other commercial reagents were used without further purification. The NMR spectra were obtained in a DMSO-d₆ solution in a Bruker AVANCE 300 spectrometer. ¹H NMR shifts were recorded relative to residual proton resonances in the deuterated solvent. IR spectra were obtained with a Thermo Scientific Nicolet is10 instrument. Ultraviolet-visible (UV-vis) spectra were recorded on a HP 8453 diode array instrument. Electrospray ionization mass spectrometry (ESI-MS) spectra were obtained using a Thermo Finnigan LXQ with methanol as the solvent. Conductivity measurements were performed with a LaMotte CDS 5000 conductimeter. Circular dichroism (CD) spectra were recorded on a Chirascan spectrometer with a 150W xenon arc lamp. Steady-state fluorescence measurements were carried out using a photon technology international (PTI), fluorescence master system A1010B arc lamp, LBS 220B lamp power supply, 814 photomultiplier detection system. Metal analysis was performed on a Perkin Elmer Optimal 3000 Inductively Coupled Plasma (ICP) emission spectrometer, samples and standards were prepared in 10% HCl. Standards were prepared diluting a 1000 mg/L platinum standard solution from Sigma-Aldrich Co, the samples were heated in a water bath at 70 °C for 30 min before analysis.

2.2. Synthesis of complexes

2.2.1. *trans*-Pt(CQDP)₂(I)₂ [1]—A solution of K₂[PtCl₄] (100 mg, 0.24 mmol) in water (30 mL) was stirred until complete dissolution was achieved, an excess (20-fold) of KI was added and finally CQDP dissolved in water (250 mg, 0.48 mmol) was added. The stirring was continued for 1 h at room temperature, and a yellow precipitate was obtained. This was collected by filtration, washed with water, and dried under vacuum. Yield 81%; Elemental analysis (%) Calc. for C₃₆H₆₄N₆Cl₂I₂O₁₆P₄Pt (1480.74 g.mol⁻¹): C 29.2; N 5.7; H 4.4. Found: C 29.9; N 5.6; H 4.2. ESI-MS (MeOH): (M- 4H₃PO₄) 1089; IR: ν (N-H) 3314 cm⁻¹; ν (C=C) 1613 cm⁻¹; ν (C=N) 1579 cm⁻¹; ν (Pt-I) 344 cm⁻¹; ν (Pt-N) 478 cm⁻¹. UV-vis

(DMSO) 262 and 345 nm. ϵ (DMSO) [λ nm]: 54700 M⁻¹cm⁻¹ (261 nm) and 25800 M⁻¹cm⁻¹ (348 nm). ¹H-NMR (DMSO-d₆; δ ppm): 8.86 (1H; d; J, 7.71 Hz; NH); 8.65 (1H; d; J, 9.18 Hz; H₅); 8.59 (1H; d; J, 7.08 Hz; H₂); 7.90 (1H; d; J, 1.68 Hz; H₈); 7.82 (1H; dd; J₁, 1.56 Hz; J₂, 9.03 Hz; H₆); 7.00 (1H; d; J, 7.26 Hz; H₃); 4.16 (1H; m; H₁'); 3.10 (6H, m, H₄' and H₅'); 1.72 (4H; m; H₂' and H₃'); 1.31 (3H; d; J, 6.21 Hz; H₁''); 1.16 (6H, t, H₆'); ¹³C-NMR (DMSO-d₆; δ ppm): 155.43 (C₄); 143.43 (C₂); 138.99 (C₉); 138.71 (C₇); 127.33 (C₆); 126.31 (C₅); 119.6 (C₈); 115.88 (C₁₀); 99.35 (C₃); 51.14 (C₄'); 49.72 (C₁'); 46.99 (C₅'); 32.48 (C₂'); 20.66 (C₃'); 20.02 (C₁''); 9.14 (C₆'). ³¹P-NMR (DMSO-d₆; δ ppm): -0.40 (H₂PO₄⁻). Molar conductivity in Dimethylformamide (DMF), $\Lambda_M = 299 \pm 15$ ohm⁻¹ cm² mol⁻¹.(1)

2.2.2. trans - Pt(CQDP)₂(Cl)₂ [2]—A solution of K₂[PtCl₄] (100 mg, 0.24 mmol) in water (30 mL) was stirred until complete dissolution was achieved and then CQDP (250 mg, 0.48 mmol) was added. The stirring was continued for 12 h at room temperature, and a pink precipitate was obtained. This was collected by filtration, washed with water, and dried under vacuum. Yield 88%; Elemental analysis (%) Calc. for C₃₆H₆₄N₆Cl₄O₁₆P₄Pt (1297.65 g.mol⁻¹): C 33.3; N 6.5; H 4.9. Found: C 32.5; N 6.4; H 5.0. ESI-MS (MeOH) (M-4H₃PO₄) 905.28 m/z; IR ν (N-H) 3331 cm⁻¹; ν (C=C) 1612 cm⁻¹; ν (C=N) 1583 cm⁻¹; ν (Pt-I) 317 cm⁻¹; ν (Pt-N) 422 cm⁻¹. UV-vis 240 and 344 nm. ϵ (DMSO) [λ nm]: 31600 M⁻¹cm⁻¹ (262 nm) and 34700 M⁻¹cm⁻¹ (348 nm). ¹H-NMR (DMSO-d₆; δ ppm): 9.12 (1H; d; J, 8.10 Hz; NH); 8.85 (1H; d; J, 9.15 Hz; H₅); 8.54 (1H; d; J, 7.10 Hz; H₂); 8.04 (1H; d; J, 1.95 Hz; H₈); 7.73 (1H; dd; J₁, 1.90 and J₂, 9.10 Hz; H₆); 6.98 (1H; d; J, 7.30 Hz; H₃); 4.14 (1H; m; H₁'); 3.06 (6H, m, H₄' and H₅'); 1.76 (4H; m; H₂' and H₃'); 1.31 (3H; d; J, 6.30 Hz; H₁''); 1.18 (6H, t, H₆'); NMR-¹³C (DMSO-d₆; δ ppm): 155.44 (C₉); 143.27 (C₂); 139.03 (C₄); 138.40 (C₇); 127.04 (C₆); 126.74 (C₅); 119.36 (C₈); 115.89 (C₁₀); 99.23 (C₃); 50.84 (C₄'); 49.79 (C₁'); 46.63 (C₅'); 32.43 (C₂'); 20.51 (C₃'); 20.03 (C₁''); 8.92 (C₆'). ³¹P-NMR (DMSO-d₆; δ ppm): 0.00 (H₂PO₄⁻). Molar conductivity in DMF, $\Lambda_M = 311 \pm 15$ ohm⁻¹ cm² mol⁻¹.(2)

2.2.3. trans-Pt(CQ)₂(Cl)₂ [3]—A suspension of K₂[PtCl₄] (41.9 mg, 0.1 mmol) in water (20 mL) was refluxed to complete dissolution, after addition of Ag(CH₃COO) (80.3 mg; 0.48 mmol) a white precipitated was obtained. The AgCl was filtrated and chloroquine (0.68 g, 0.25 mmol) in methanol was added to the solution, the mixture was stirred and refluxed for 30 min. Finally, KCl was added in excess to displace the acetate groups, leaving a yellow precipitate that was filtered off, washed with water and diethyl ether, and dried under vacuum. Yield 63%; Elemental analysis (%) Calc. for C₃₆H₅₂N₆Cl₄Pt (906.91 g.mol⁻¹): C 47.7; N 9.3; H: 5.8. Found: C 47.1; N 8.8; H 5.1. ESI-MS (MeOH) (M+H⁺) 906.3; IR ν (N-H) 3328 cm⁻¹; ν (C=C) 1612 cm⁻¹; ν (C=N) 1582 cm⁻¹; ν (Pt-Cl) 336 cm⁻¹; ν (Pt-N) 348 cm⁻¹. UV-vis 221 and 343 nm. ϵ (DMSO) [λ nm]: 107990 M⁻¹cm⁻¹ (262 nm) and 63150 M⁻¹cm⁻¹ (347 nm). ¹H-NMR (DMSO-d₆; δ ppm): 8.49 (1H; d; J, 9.03 Hz; H₅); 8.36 (1H; d; J, 5.43 Hz; H₂); 7.77 (1H; d; J, 1.95 Hz; H₈); 7.40 (1H; dd; J₁, 1.95 and J₂, 9.00 Hz; H₆); 7.15 (1H; d; J, 7.38 Hz; NH); 6.49 (1H; d; J, 5.61 Hz; H₃); 3.72 (1H; m; H₁'); 2.82 (6H; m; H₄' and H₅'); 1.66 (4H; m; H₂' and H₃'); 1.21 (3H; d; J, 6.27 Hz; H₁''); 1.17 (6H; t; H₆'). ¹³C-NMR (DMSO-d₆; δ ppm): 152.23 (C₂); 150.91 (C₄); 148.99 (C₉); 134.86 (C₇); 127.20 (C₈); 125.16 (C₅); 124.82 (C₆); 118.04 (C₁₀); 99.60 (C₃); 51.76 (C₅'); 48.44 (C₁'); 46.90 (C₄'); 33.32 (C₂'); 21.67 (C₃'); 20.33 (C₁''); 9.80 (C₆'). Molar conductivity in DMF, $\Lambda_M = 11 \pm 2$ ohm⁻¹ cm² mol⁻¹.(3)

(1)Although the elemental analyses values for "C" for compound 1 are somewhat unsatisfactory, the ESI-MS results and IR, ¹H and ¹³C NMR spectroscopic analysis data reasonably support the formula of these compounds

(2)Although the elemental analyses values for "C" for compound 2 are somewhat unsatisfactory, the ESIMS results and IR, ¹H and ¹³C NMR spectroscopic analysis data reasonably support the formula of these compounds

2.3. DFT Calculations

All calculations and geometry optimizations were performed with the Gaussian03 package program [23] at DFT level using B3PW91 density functionals. The all-electron 6–201331+G basis sets for C and H, the 6–31+G(d) for Cl and N, and the LANL2DZ effective core potential for Pt [24] with its corresponding atomic basis sets were employed. The p function set of the LANL2DZ basis set was uncontracted in a contraction scheme [431/3111/111]. This was done in order to obtain a greater flexibility in the p functions set to represent the empty $6p$ orbital. Although this orbital is unoccupied, it is well known that in general the empty $(n+1)p$ orbitals play an important role in the structure of transition metal complexes. Frequency calculations of all structures showed that all frequencies were positive indicating that all structures are real minima.

2.4. DNA interaction studies

In the covalent binding studies, the platinum complexes were mixed with CT-DNA and incubated for 72 h (Ri 0.2, 1 mL of metal complex and 1 mL DNA). DNA was precipitated by adding EtOH (2X sample volume) and 2 M NaCl (0.1X sample volume). After centrifugation, the supernatant was removed and the DNA was resuspended in water overnight. This precipitation–resuspension cycle was repeated three times and the final suspension was analyzed for Pt by ICP atomic emission spectrometry and for DNA by the Burton assay [25].

The spectrophotometric titrations were carried out by stepwise additions of a CT DNA solution (1 mM, in 5 mM Tris-HCl, pH 7.2 and 50 mM NaCl buffer) to a solution of each complex ($\sim 70 \mu\text{M}$) in DMSO, then recording the UV–vis spectra at 330 and 343 nm after each addition. The absorption of DNA was subtracted by adding the same amounts of CT DNA to the blank. The binding affinities were obtained by using the Scatchard equation, $r/C_f = K(n-1)$, for ligand-macromolecule interactions with non-cooperative binding sites [26, 27, 28, 29], where r is the number of moles of Pt complex bound to 1 mol of CT DNA (C_b/C_{DNA}), n is the number of equivalent binding sites, and K is the affinity of the complex for those sites. Concentrations of free (C_f) and bound (C_b) complex **1** were calculated from $C_f = C(1-\alpha)$ and $C_b = C-C_f$, respectively, where C is the total Pt concentration. The fraction of bound complex (α) was calculated according to $\alpha = (A_f - A)/(A_f - A_b)$, where A_f and A_b are the absorbance of the free and fully bound complex at the selected wavelength, and A is the absorbance at any given point during the titration. K_b is obtained from the slope of the plot [30].

To measure the interaction of each complex with CT-DNA by fluorimetric titration, the excitation and emission wavelengths for the complex were set to 343 and 380 nm, respectively. Using standard right-angle emission optics, we recorded fluorescence intensity measurements using the photon counting mode and corrected for any fluctuations of the 450-W xenon arc lamp source by deflecting a portion of the excitation signal onto a separate photodiode. The fluorimetric titration was carried out at room temperature. The complex was dissolved in a buffer consisting of 70% DMSO and 30 % Tris-HCl (5 mM Tris-HCl and 50 mM NaCl; pH 7.4) to obtain a 700 μM solution. Twenty μL of that stock solution were then diluted with 1,980 μL of the same buffer in a quartz cuvette and then titrated with 10 μL additions of a 90 μM solution of CT DNA (5 mM Tris-HCl {pH 7.2}, 50 mM NaCl). Emission spectra were monitored at 380 and 550 nm until saturation was reached. The binding affinities were obtained using the Scatchard equation $r/C_f = K(n-1)$.

⁽³⁾Although the elemental analyses values for "C" and "H" for compound **3** are somewhat unsatisfactory, the ESI-MS results and IR, ^1H and ^{13}C NMR spectroscopic analysis data reasonably support the formula of these compounds

Viscosity measurements were carried out using an Ostwald viscometer immersed in a water bath maintained at 25 °C. The DNA concentration (75 µM in 5 mM Tris-HCl {pH 7.2}, 50 mM NaCl) was kept constant in all samples, while the complex concentration was increased from 0 to 67 µM. The flow time was measured at least 6 times with a digital stopwatch and the mean value was calculated. Data are presented as $(\eta/\eta^0)^{1/3}$ versus the ratio [complex]/[DNA], where η and η^0 are the specific viscosity of DNA in the presence and absence of the complex, respectively. The values of η and η^0 were calculated by use of the expression $(t - t^b)/t^b$, where t is the observed flow time and t^b is the flow time of buffer alone. The relative viscosity of the DNA was calculated from η / η^0 [31].

For DNA electrophoresis assays, 10 µL samples of the plasmid pBR322 (20 µg/mL) were combined with the complex at different ratios and then incubated for 18 h at 37 °C. Five µL of each sample were run (100 mV for 45 min) on a 1% agarose gel with TBE-1X (0.45 M Tris-HCl, 0.45 M boric acid, 10 mM EDTA) and stained with ethidium bromide (5 µL ethidium bromide per 50 mL agarose gel mixture). The bands were then viewed with a trans-luminator and the image captured with a camera [32].

For the circular dichroism measurements, a solution of each complex was freshly prepared in DMSO (5 mM). The appropriate volumes of that solution were added to 3 mL samples of a freshly prepared solution of CT DNA (195 µM) in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, pH=7.29) to achieve molar ratios of 0 – 0.5 drug/DNA. The samples were incubated at 37°C for 18 h. All CD spectra of DNA and of the DNA-drug adducts were recorded at 25°C over the range 220–330 nm and finally corrected with a blank and using noise reduction. The final data is expressed in molar ellipticity (millidegrees) [33].

2.5. Growth inhibition and cytotoxicity testing

Five human and one murine tumour cell lines were used. HT-29 and LoVo (human colon carcinoma), MCF-7 and SKBR-3 (human breast carcinoma), PC-3 (human prostate carcinoma) and B16/BL6 (murine melanoma) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine, (Gibco, BRL, USA) and penicillin (100 Units/mL) – streptomycin (100 µg/mL), containing in addition glucose 0.45% for the HT-29 cells. The sulphorhodamine B (SRB) assay was used to evaluate the effect of the compounds on the growth and viability of the six tumour cell lines [34]. Each drug was assayed in triplicate at 6 different concentrations up to a maximum of 30 µM. The concentrations inducing 50% growth inhibition (GI₅₀), total growth inhibition (TGI) and 50% cytotoxicity (LC₅₀) after a 48 h incubation period were calculated by linear interpolation from the observed data points.

3. Results and discussion

3.1. Synthesis and characterization

3.1.1. Pt-CQDP complexes—The new platinum-chloroquine diphosphate complexes **1** and **2** were synthesized at room temperature by the reaction of K₂[PtX₄] with CQDP in water (Fig. 1). In the case of (**1**), CQDP displaced two labile iodide ligands, while in (**2**) CQDP displaced two chloride ligands. Elemental analyses of these complexes are in agreement with the molecular formula proposed. The IR spectra of the complexes displayed peaks clearly associated with the presence of the coordinated CQDP. The far-IR spectrum (S1) showed bands at 344 and 317 cm⁻¹ attributable to Pt–I and Pt–Cl vibrations for complexes **1** and **2** respectively. The presence of only one band in this region supported the assignment of the *trans* geometry for these complexes [35]. Additionally a characteristic Pt–N vibration band appears at 478 cm⁻¹ and 422 cm⁻¹ for complexes **1** and **2** respectively [36,37,38]. The ESI-MS spectrum of complex **1** displayed parent peaks of high intensity

corresponding to its molecular ion ($M - 4H_3PO_4$) at m/z 1089.03, while complex **2** showed one high intensity ion peak at m/z 905.28 corresponding to $M - 4H_3PO_4$. The molar conductivity values obtained for the complexes **1** and **2** are in the range for 1:4 electrolytes dissolved in DMF [39], corresponding to four phosphates ($H_2PO_4^-$) of CQDP in each platinum complex. All NMR signals could be unequivocally assigned on the basis of 1D and 2D, Correlation spectroscopy (COSY), Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments for both complexes (for complete NMR data see Experimental Section; atom numbering for CQDP in Figure 1). The 1H and ^{13}C chemical shift variation of each signal with respect to those of the free ligand ($\Delta\delta$) was used as a parameter to deduce the mode of bonding of CQDP to the metal. It has been previously shown by us [20] and by others [18] that the largest variations are always observed for the protons and carbons located in the vicinity of the N-atom attached to the metal. In complex **1** and **2**, the largest shift with respect to the free ligand (CQDP) was observed for NH and H1' in the 1H NMR spectra and C4 in the ^{13}C NMR spectra (Table 1). All other chloroquine protons and carbons showed smaller displacements, indicating that CQDP is bound to the platinum through the NH atom of the secondary amine, a good donor site in this molecule. Additionally, one signal was observed in the ^{31}P -NMR corresponding to the $H_2PO_4^-$ group of CQDP (see experimental section). According to the available data, the formulation for the new platinum-chloroquine diphosphate compounds corresponds to 16-electron Pt(II) complexes in the usual d^8 square planar coordination geometry, of *trans* configuration due to steric repulsion between the two chloroquine diphosphate ligands.

3.1.2. *trans*-Pt(CQ)₂(Cl)₂ (3**)**—The reaction of $K_2[PtCl_4]$ with 4 eq of $AgCH_3COO$ in water/methanol followed by treatment with CQ was the most efficient way to prepare $[Pt(CQ)_2(Cl)_2]$ (**3**), which was isolated in good yields as a yellow solid. While our work was in progress, the same compound was independently synthesized by the group of Ajibade and Kolawole by a slightly different procedure [40]. As in the case of the other complexes, the characterization has been based on elemental analyses, IR, ESI-MS spectroscopy, and mainly NMR spectroscopy, which allowed assignment of all the resonances. Elemental analyses of complex **3** are in agreement with the molecular formula proposed. The ESI-MS spectrum for complex **3** displays the molecular ion peak ($M+H^+$) at m/z 906.28 with high intensity. The IR spectra of the complex displayed peaks clearly associated with the presence of the coordinated ligand and one band in the far-IR regions at 336 cm^{-1} attributable to the Pt-Cl vibration. Also, a characteristic Pt-N vibration band appears at 384 cm^{-1} supporting the assignment of the *trans* geometry [35]. On the basis of the data shown in Table 1, we propose that CQ binds to the platinum in complex **3** through the tertiary amine, since large shifts with respect to free CQ were observed for H_4' y H_5' ($\Delta\delta = 0.53$), C_4 ($\Delta\delta = 5.66$), and C_5 ($\Delta\delta = 5.14$), while all other protons and carbon signals suffered minor displacements with respect to the free ligand. These findings suggest that complex **3** is non-electrolytic, which was verified by the corresponding molar conductivity values, which were below the expected range for electrolyte 1:1 [39]. The formulation for complex **3** also corresponds to a 16-electron Pt(II) complex in the usual d^8 square planar coordination geometry, most probably in *trans* configurations due to steric repulsion between the two CQ ligands.

3.2. Theoretical calculations

In order to provide further support for our structural proposals based on spectroscopic data, we have also established the relative stability of the different possible isomers of $Pt(CQDP)_2(Cl)_2$ by performing DFT calculations on a $[Pt(H_2CQ)_2(Cl)_2]^{+4}I^{-1}$ ($H_2CQ = C_{18}H_{28}N_3Cl$) model compound. The $H_2PO_4^-$ counterions were substituted by I^- in order to reduce the computational cost. Although the ionic radius of I^- ($2.16\text{ \AA}$) is smaller than the

estimated $\text{H}_2\text{PO}_4^{-1}$ radius (3.2 Å), we believe that I^- is large and soft enough to account for all important electrostatic interactions. Four structural isomeric forms were found; in all isomers the Pt atom has a square planar structure with a Pt-Cl distance of 2.36 Å. Table 2 shows the relative energy between the isomers and the Pt-N distances. Isomer **A** (Figure 2a) is the most stable and corresponds to the Pt atom interacting with the N atom of the secondary amine. Isomer **B**, interacting through the quinoline N atom, is less stable than **A** due to the Pt-N interaction forcing the N atom to change his configuration from planar sp^2 to tetrahedral sp^3 ; this moves the N atom out of the ring plane and partially destroys ring aromaticity. Isomer **C**, the least stable, corresponds to the Pt atom interacting with the N of the tertiary amine. This structure has the largest Pt-N distance (2.18 Å), which is an indication of severe steric crowding around this site.

Cis- and *trans*- $\text{Pt}(\text{CQ})_2(\text{Cl})_2$ isomers were also studied using DFT calculations. The theoretical results show that the lower energy structure corresponds to the *trans*-isomer (Figure 2b) where the Pt atom is bound through the tertiary amine to the CQ ligand, in agreement with the experimental results.

3.3. DNA interaction studies

Pt complexes are known to bind covalently to DNA, which is the basis of its anticancer activity [41]. Also, chloroquine has been shown to bind to DNA through intercalation and electrostatic interactions and this mode of binding is retained in the case of Ru-CQ complexes through the coordinated CQ moiety, although it is not clear if such DNA binding is responsible for their antitumor activity [10, 11]. It is therefore important to establish whether the new Pt-CQ and Pt-CQDP derivatives bind effectively to DNA and what types of interactions are playing the major roles.

The results of covalent binding studies for complexes **1–3** shown in Table 3 indicate that while **1** and **2** bind around 0.12 Pt atom/ bases, **3** is bound 0.47 Pt atom/bases (corresponding to two bases/Pt). These binding levels are comparable to those observed for rhodium complexes [42] and this can be taken as evidence that some Pt-DNA covalent binding is taking place with the new complexes, and in a noticeably stronger manner for complex **3**. The levels of covalent binding of cisplatin and transplatin with DNA measured by us, also included in Table 3 for comparison, are in agreement with previously reported values [43,44] and are higher than the ones measured for **1–2** but comparable to **3**.

In order to shed further light into the mode of Pt-DNA interactions in complexes **1–3**, we performed absorption and emission titration experiments. The absorption plots showed that adding DNA to solutions of each complex to saturation caused hypochromism at the absorbance maxima (330 and 343 nm), and two isosbestic points at 290 and 350 nm. As an example, the data for complex **1** are shown in Fig. 3; the corresponding binding constants (K_b) for all the complexes are collected in Table 4. The values lie within the interval for which a compound is considered to be interacting with DNA [45,46] and for complexes **1** and **2** are very similar to those for CQ, while for **3** they are somewhat higher. They are also comparable to K_b values obtained for other transition metal-(CQ) complexes [47,48]. The emission bands of complexes **1–3** at 389 nm decrease in intensity (Figure 4) as DNA is added until saturation. The binding constants calculated using a Scatchard plot for the data at the emission maxima are shown in Table 4. These values are consistent with those calculated from absorption studies and similar to the ones obtained for CQ and other metal-CQ complexes [20,21,49], indicating that these complexes interact with DNA in a manner analogous to free CQ. Such interactions have been described in terms of intercalation through the planar CQ moiety plus an electrostatic component between the charged complex and negatively charged phosphate residues in the nucleic acid polymer [50]. Some reversible interactions such as intercalation, hydrogen bridging or electrostatic appear to be taking

place besides the covalent interaction proposed above. Also consistent with the covalent binding results, the emission data seem to indicate that the interaction of complex **3** is stronger than the corresponding ones for free CQ or complexes **1** and **2**.

CD spectroscopy has been widely used to examine changes in DNA morphology during drug–DNA interactions, as the band due to base stacking (275 nm) and that due to right-handed helicity (248 nm) are quite sensitive to the mode of DNA interactions with small molecules [51]. Figure 5 shows the spectra of all complexes evaluated with CT DNA solutions at different ratios, as well as the CD spectrum of DNA alone. Complexes **1** and **2** do not produce significant changes in the ellipticity values indicating that they do not modify the DNA tertiary structure, while complex **3** is able to decrease the ellipticity of the positive band and caused a slight increase and blue shift of the negative band (Figure 5). These modifications could be attributable to complex **3** binding covalently to DNA. [32].

Circular plasmid DNA is ideally suited to probe cleavage events as DNA exists in a supercoiled state in its native form and converts to a relaxed form upon single strand cleavage, exhibiting an altered migration rate in agarose gel electrophoresis [52, 53]. Addition of the three complexes to pBR322 plasmid DNA led to changes in the mobility of the plasmid. Figure 6 shows the electrophoresis of the plasmid in the presence of different molar ratios of complexes **1** and **3** (complex **2** showed similar behavior, results not shown). Line 2 displays the difference in mobility of the plasmid alone (control line), line 3 corresponds to the plasmid incubated with cisplatin, while lines 4–6 correspond to the plasmid incubated with different concentrations of the complexes. The increase in the concentration of each platinum complex caused changes in the mobility of the plasmid. Two bands are evident for complex **1** at R_i between 0.5 and 1, representing both the supercoiled and circular forms. At $R_i = 2$, only one band is observed, attributable to the circular form. It is noticeable that complex **3** at $R_i = 0.5$ (Figure 6b) displayed one band corresponding to the circular form of the plasmid and at a higher R_i , no bands are visible for DNA, either relaxed or linear.

Viscosity measurements were used to further elucidate the interaction between the complexes and DNA. Hydrodynamic measurements that are sensitive to length change are regarded as the least ambiguous and the most critical tests of a binding model in solution in the absence of crystallographic structural data. The model demands that classical intercalators, such as ethidium bromide, lengthen the DNA helix as base pairs are separated to accommodate the binding ligand, leading to an increase in DNA viscosity. In this study, compounds **1–3** show slightly changes in the relative viscosity of DNA with increasing concentrations of each complex. Similar behavior was observed with cisplatin, evaluated for us in the same experiment (S2), while the absence of a change in the relative viscosity of DNA suggested that these complexes, like cisplatin, do not engage in DNA intercalation.

Analyzing all the results of our DNA binding studies together, we suggest that compounds **1** and **2** interact with DNA primarily through electrostatic contacts and hydrogen bonding, with a minor contribution of a covalent interaction. It is likely that for these compounds a more marked non-covalent interaction is observed because the positive charges on the CQDP ligand promote electrostatic interactions with the phosphates on the nucleic acid and/or hydrogen bonding. Complex **3**, on the other hand, displayed mainly covalent binding with DNA; it is possible to envisage a predominantly transplatin-like mechanism involving aquation of one or both chlorides, followed by covalent bonding between the metal and a nucleobase, most likely guanine.

3.4. Growth inhibition and cytotoxicity

The compounds were tested on six human tumor cell lines, representing tumors of three different origins, prostate, breast and colon, in addition to a murine melanoma line, which we regularly use for *in vivo* testing of anticancer drugs that show promising results *in vitro*. The results are shown in Table 5. As many drugs show a cytostatic effect at doses appreciably lower than those causing cytotoxicity, we used the SRB assay which has the advantage over tetrazolium assays of being able to distinguish between a cytostatic effect, where the drug decreases the rate of cell proliferation and a cytotoxic effect which represents a true decrease in the number of viable cells [54]. These and other advantages have made it the method of choice for drug screening at the National Cancer Institute (USA) for the last 20 years [55].

Although all three compounds exerted some degree of growth inhibition on the human tumor cell lines, complex **3** was evidently the most active, showing total growth inhibition on all the cell lines and a cytotoxic effect on two of them at concentrations below 30 μ M. This activity was greater than that shown by the control CQ and platinum compounds. The low activity of cisplatin seen in these assays, when compared to its well-known cytotoxicity as reported in the literature, is probably due to the relatively short incubation times used here (48 h). It is interesting that the cytotoxic activity of complex **3** correlates with the strong interaction observed between this complex and DNA, which was similar to that shown for cisplatin. This may suggest that this complex is in fact exerting its cytotoxic effect through an interaction with DNA although further experiments must be performed to confirm this hypothesis.

4. Conclusions

The synthesis and characterization by of two new trans- platinum-chloroquine diphosphate (**1** and **2**) and one trans- platinum-chloroquine (**3**) complexes were achieved. Complexes **1** and **2** are proposed to interact with DNA mainly through electrostatic contacts and hydrogen bonding, with a minor contribution of a covalent interaction, while complex **3** interacts with the DNA mainly by covalent binding. All compounds exerted some degree of growth inhibition on the human tumor cell lines, with complex **3** showing the most promising results, a greater activity than those shown by CQ and platinum compounds (transplatin and cisplatin) under these experimental conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Complex 1

Complex 2

i

ii

iii

Chloroquine base (CQ)

iv

Complex 3

Fig. 1.

Synthesis of new chloroquine–platinum complexes: (i) $\text{K}_2[\text{PtCl}_4]/\text{KI}/\text{CQDP}$ 1:20:2 in water, rt; (ii) $\text{K}_2[\text{PtCl}_4]/\text{CQDP}$ 1:2 in water, rt; (iii) $\text{NH}_4\text{OH}/\text{H}_2\text{O}/(\text{CH}_3\text{CH}_2)_2\text{O}$; (iv) $\text{K}_2[\text{PtCl}_4]/\text{Ag}(\text{CH}_3\text{COO})/\text{CQ}/\text{KCl}$ 1:4:2:excess in water/methanol, reflux.

Fig. 2. Optimized structures for complexes **2** (a) and **3** (b). Purple spheres - I atoms. Green sticks (*)- Cl atoms

Fig. 3. Spectrophotometric titration spectra of Pt(CQDF)₂(I)₂ with CT-DNA. [Complex] = 6.07×10^{-6} M, [DNA] = 0–150 μ M.

Fig. 4. Fluorimetric titration spectra of $\text{Pt}(\text{CQDF})_2(\text{I})_2$ with CT-DNA. $[\text{Complex}] = 6.07 \times 10^{-6} \text{ M}$, $[\text{DNA}] = 0\text{--}150 \text{ }\mu\text{M}$.

Fig. 5. CD spectra versus of complexes **1–3** at different $[\text{complex}]/[\text{DNA}]$ ratios. (a) $[\text{Pt}(\text{CQDP})_2(\text{I})_2]/[\text{DNA}]$ (b) $[\text{Pt}(\text{CQDP})_2(\text{Cl})_2]/[\text{DNA}]$ (c) $[\text{Pt}(\text{CQ})_2(\text{Cl})_2]/[\text{DNA}]$.

Fig. 6.

Effects of varying concentrations of complexes 1–3 on the conformation of pBR322 plasmid DNA (a) $[\text{Pt}(\text{CQDP})_2(\text{I})_2]/[\text{DNA}]$ (b) $[\text{Pt}(\text{CQ})_2(\text{Cl})_2]/[\text{DNA}]$. The R_i values are relation complex:DNA. (1) Molecular weight marker (2) DNA in DMSO (3) Cisplatin - DNA R_i 1 (4) Complex - DNA R_i 0.5 (5) Complex - DNA R_i 1 (6) Complex - DNA R_i 2

Table 1

Displacement of protons and carbons ($\Delta\delta$, ppm) of the CQDP and CQ groups in complexes **1–3** with respect to the free ligands (DMSO as solvent).

	Complex				Complex			
	Protons	1	2	3	Carbons	1	2	3
H6'	0.25	0.13	0.26		C2	7.93	8.29	0.07
H1''	0.07	0.08	0.07		C4	9.71	9.67	0.94
H2' and H3'	0.13	0.12	0.21		C9	4.85	4.76	0.78
H4' and H5'	0.30	0.24	0.53		C7	4.43	4.12	1.11
H1'	0.43	0.38	0.10		C8	7.48	7.72	0.69
H3	0.48	0.45	0.08		C6	2.74	2.45	0.99
H6	0.36	0.30	0.06		C5	1.12	1.56	0.04
H8	0.15	0.27	0.08		C10	1.98	1.97	0.07
H2	0.20	0.17	0.07		C3	0.02	0.10	0.36
H5	0.30	0.46	0.05		C5'	0.49	0.12	5.14
NH	1.97	2.04	0.32		C1'	1.59	1.67	0.39
					C4'	0.27	0.56	5.66
					C2'	0.64	0.69	0.50
					C3'	0.79	0.93	2.24
					C1''	0.25	0.24	0.07
					C6'	0.48	0.69	2.30

Table 2Energy differences (in kcal/mol) between isomeric forms for complex **1**

Isomer	Relative Energy (kcal/mol)	Pt-N distance (Å)
A	0.00	2.10
B	+29.5	2.11
C	+41.8	2.18

Table 3

Covalent binding values for the new platinum-chloroquine complexes

Complex	nmol Pt / mg DNA (metal/base)
(1) Pt(CQDP) ₂ (I) ₂ ,	183.41 ± 4.54 (0.12 ± 0.01)
(2) Pt(CQDP) ₂ (Cl) ₂	171.72 ± 25.62 (0.11 ± 0.02)
(3) Pt(CQ) ₂ (Cl) ₂	721.49 ± 24.09 (0.47 ± 0.04)
<i>cis</i> -Pt(NH ₃) ₂ (Cl) ₂	1136.52 ± 15.78 (0.74 ± 0.09)
<i>trans</i> -Pt(NH ₃) ₂ (Cl) ₂	1135.47 ± 14.12 (0.74 ± 0.01)

Table 4

Binding constants for the interaction between platinum complexes 1–3 and calf thymus DNA.

Complex	Absorption titration		Emission titration	
	$K_{b1}(\times 10^7 \text{ M}^{-1})$	$K_{b2}(\times 10^5 \text{ M}^{-1})$	$K_{b1}(\times 10^7 \text{ M}^{-1})$	$K_{b2}(\times 10^5 \text{ M}^{-1})$
Pt(CQDP) ₂ (I) ₂	0.71 ± 0.20	1.14 ± 0.15	0.67 ± 0.21	3.07 ± 0.82
Pt(CQDP) ₂ (Cl) ₂	0.53 ± 0.11	3.68 ± 0.92	0.14 ± 0.01	2.27 ± 0.12
Pt(CQ) ₂ (Cl) ₂	3.50 ± 0.92	5.77 ± 0.98	4.25 ± 0.06	4.50 ± 1.04
CQDP	1.38 ± 0.55	0.93 ± 0.21	3.24 ± 1.21	3.26 ± 1.01

Table 5

Cytostatic and cytotoxic effects of the compounds against six tumor cell lines.

Complexes	PC-3			MCF-7			SKBR-3			HT-29			LoVo			BI6/BL6		
	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀	GI ₅₀	TGI	LC ₅₀
Pt(CQDP) ₂ (I) ₂ (1)	13	>30	>30	>30	>30	>30	>30	>30	>30	16	24	>30	7	20	>30	16	>30	>30
Pt(CQDP) ₂ (Cl) ₂ (2)	10	>30	>30	30	>30	>30	>30	>30	>30	15	24	>30	7	>30	>30	12	20	28
Pt(CQ) ₂ (Cl) ₂ (3)	7	19	>30	8	22	>30	8	13	24	7	10	24	6	14	>30	9	19	>30
CQ	20	>30	>30	>30	>30	>30	>30	>30	>30	23	>30	>30	10	27	>30	28	>30	>30
CQDP	17	>30	>30	>30	>30	>30	>30	>30	>30	20	29	>30	8	30	>30	20	>30	>30
Cisplatin	>30	>30	>30	26	>30	>30	6	23	>30	>30	>30	>30	>30	>30	>30	25	>30	>30
Transplatin	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30

GI₅₀ - 50% growth inhibition, TGI - total growth inhibition, LC₅₀ - 50% cytotoxicity. CQ - Chloroquine, CQDP - Chloroquine diphosphate. Concentrations expressed in μM.