Platinum(II) compounds with chelating ligands based on pyridine and pyrimidine: DNA and protein binding studies

Roy, S.; Westmaas, J.A.; Hagen, K.; Wezel, G.P. van; Reedijk, J.

Journal of Inorganic Biochemistry 103 (2009) 1288–1297

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Platinum(II) compounds with chelating ligands based on pyridine and pyrimidine: DNA and protein binding studies

Sudeshna Roy, Joyce A. Westmaas, Katharine D. Hagen, Gilles P. van Wezel, Jan Reedijk *

Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 17 April 2009
Received in revised form 30 June 2009
Accepted 6 July 2009
Available online 6 August 2009

Keywords:
Platinum
Flexible amines
Antitumor
DNA cleavage
CD
BSA
Lipophilicity

ABSTRACT

Four Pt(II) compounds (**C1**–**C4**) have been studied in their DNA and protein binding. The compounds contain chelating diimine ligands bis(pyridine-2-yl)amine, abbreviated **dpa**, and bis(pyrimidine-2-yl)amine, abbreviated **dipm**. The anions for the compounds **C1** and **C3** are chloride (coordinated) and nitrate (non-coordinated) for **C2** and **C4**. Calf-thymus DNA and an abundant plasma protein have been taken as models for the two major targets for metallodrug interactions investigated by CD spectroscopy. The modifications in the carrier ligands (chloride or ammine) or ancillary secondary amines have been considered to reveal the mode of interactions. The simultaneous effects of coordinative binding and partial intercalation to DNA are evident from several spectroscopic studies. To evaluate the permeability of the cytoplasmic and cellular membrane and the transportation inside the cells, partition coefficients of the four platinum compounds were determined. Two compounds (**C3** and **C4**) induce two-step single-strand DNA cleavage, initiated by partial intercalation. The combined effect of several binding modes towards different bio-molecules is elucidated, providing a rationale for their *in vitro* activity profile.

1. Introduction

Genomic DNA is known as the main target for platinum antitumour compounds, such as cisplatin, carboplatin and oxaliplatin. In addition, several cellular components other than DNA act as platinophiles (*e.g.* glutathione and metallothionein) and cellular proteins comprise of a significant part of the platinum-deactivating agents. Besides the cytotoxicity assay for the new platinum compounds, the interaction with biological targets plays a crucial role to elucidate the mode of action and to find specific directions to improve the activity profile.

DNA is a chiral molecule which is polymorphic in secondary structure and which can be perturbed by several solution properties or presence of external molecules (*e.g.* a platinum compound or intercalating ligands) [1,2]. The typical B-form DNA gives rise to two conservative CD bands in the UV-region. The positive band at 278 nm, which originates from base stacking and the negative band at 246 nm, which arises from the right-handed helicity of DNA are the two characteristic bands. Upon addition of a metal compound or a ligand to the B-DNA solution, changes in these two bands are commonly recorded and plotted with the native DNA spectrum, which are indicative for the mode of interaction [3].

The coordination binding of cisplatin with the DNA inhibits replication both *in vitro* and *in vivo*, and the antiproliferative property of cisplatin originates from DNA binding and distortion in DNA structure [4–7]. The other platinum drugs such as carboplatin and oxaliplatin are also assumed to exhibit antitumour activity, similar to cisplatin, *via* DNA binding. A few platinated-DNA crystal structures have been reported [8–17]. Other than the coordinative monodentate or bidentate guanosine adducts on the DNA strand, intercalation, groove-binding or electrostatic binding modes have been observed for platinum compounds [18–21]. Therefore, up to now DNA has been accepted to be the prime intracellular target for cisplatin-like metallodrugs. For other platinum coordination compounds the binding mode is not known *a priori*.

UV–vis spectroscopy, based on the absorption of photons of monochromatic light, is applicable in bio-physical studies to elucidate DNA binding properties. When a platinum compound binds to DNA by coordination, hyperchromicity (increase in absorption of the DNA band at 270 nm) is observed [22,23]. When intercalation takes place between a compound and DNA base pairs, hypochromism and a red shift is observed due to the strong π–π-stacking interaction between the DNA base pairs and the aromatic part of the compound [22,24,25].

Albumin is the most abundant protein in the plasma and as a carrier protein it is responsible for the transport of a variety of compounds, including vitamins, fatty acids and pharmaceuticals [26]. Interactions between metallodrugs and albumin indicates the overall distribution, excretion and differences in activity, toxicity and efficacy of these compounds [27]. Cisplatin can efficiently bind to albumin, since it has a high affinity for the sulfur-rich do-

* Corresponding author.
E-mail address: reedijk@chem.leidenuniv.nl (J. Reedijk).

0162-0134/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2009.07.003

mains. Consequently, this leads to Pt–methionine adducts, which may act as a platinum reservoir for subsequent DNA platination. Therefore, elucidation of protein-platinum compound interaction to explain the *in vivo* efficacy or deactivation of Pt-containing drugs is important [28].

In the preceding paper [29], synthesis, characterizations, structure optimization by density functional theory (DFT) calculation and *in vitro* antiproliferative activity of four platinum coordination compounds (**C1**–**C4**) has been described; in addition model-base studies for **C1** and **C3** were reported. In this follow-up study, DNA and protein binding interactions are described in detail. The molecular structures of these four compounds have been drawn in Fig. 1.

The cisplatin-analogues (**C1** and **C3**) have relatively labile chloride ligands and are expected to have a similar interaction with DNA as cisplatin. Firstly, the activation of the compounds is assumed *via* hydrolysis in buffer solution. Subsequently, the aquated positively charged species are attracted to negatively charged DNA-phosphate backbone by electrostatic attraction. The aromatic ligands may facilitate the interactions by sliding partially in between base pairs. The H-bonding amine ligands (**dpa** [bis(pyridine-2-yl)amine] and **dipm** [bis(pyrimidine-2-yl)amine]) can facilitate the formation of the DNA-compound adduct further.

In contrast, compounds **C2** and **C4** are positively charged and devoid of any easily hydrolysable ligands. Therefore, these cationic compounds have other possibilities to interact with DNA, namely *via* (a) electrostatic attraction, (b) hydrophobic interactions with the major or minor groove, (c) H-bonding or (d) intercalation between base pairs. The molecular structures of the two free ligands used for these studies and cisplatin have been drawn in Fig. 2.

To predict the ability of crossing the lipid bilayer of the cell-membrane *via* passive diffusion and bio-distribution of these platinum compounds, partition coefficient has been measured [30]. The partition coefficient (log *P*) is defined as the logarithmic ratio of the concentrations of a substance in a two-phase system consisting of two immiscible solvents [31–33]. When octanol and water is used as two immiscible solvents then *P* can be calculated using the following equation:

$$\text{Partition coefficient } (\log P) = \log \frac{(\text{Pt})_{\text{octanol}}}{(\text{Pt})_{\text{water}}} \quad (1)$$

The partition ratio (*P*) can be calculated as the concentration ratio of the substance distributed in the two immiscible solvent and is further used to calculate hydrophilicity or lipophilicity of the certain substance. A compound with a high positive value of log $P_{o/w}$ is lipophilic and can easily cross the cell-membrane. A negative partition coefficient indicates hydrophilicity, making the compound more likely to stay in the aqueous solution. Therefore, blood serum is the most preferred storage cellular component for the hydrophilic compound.

2. Experimental

2.1. Chemicals

The detailed description of syntheses and characterizations for four platinum compounds (**C1–C4**) has been given in previous part.

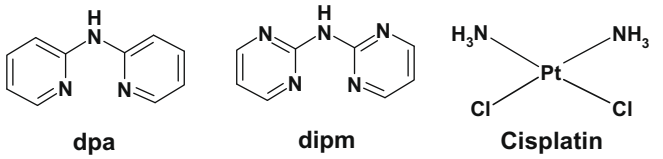


Fig. 2. Schematic structures of two ligands (**dpa** and **dipm**) and the reference compound cisplatin.

The ligands, **dpa** was obtained from Sigma Aldrich B.V. (The Netherlands) and **dipm** has been synthesized following the reported synthetic route with slight modifications [34]. Cisplatin was synthesized according to the reported literature [35]. Sodium chloride, sodium dihydrogenphosphate (NaH_2PO_4) and *n*-octanol were purchased from Merck, Germany. Disodium hydrogenphosphate (Na_2HPO_4) was obtained from ACF Chemiefarma NV (The Netherlands) and the water was of MilliQ quality from Millipore, USA. Ethidium bromide (EthBr), bovine serum albumin (BSA) and calf thymus DNA (CT DNA) were purchased from Sigma Aldrich B.V. (The Netherlands). CT DNA was purified following a typical method of size-exclusion chromatography and lyophilization. The buffer used was 10 mM phosphate buffered saline (PBS) with 50 mM NaCl. The stock solutions for the studied compounds were prepared by dissolving the compounds either in dmso (**C1** and **C3**) or in milliQ water (cisplatin, **C2** and **C4**) of a concentration of 0.5 M and subsequent dilution in phosphate buffer prior to the experiments has been carried out.

2.2. UV–vis spectroscopy

Spectra were recorded at room temperature using a Varian Cary 50 Scan UV–Visible Spectrophotometer. Quartz cuvettes with 1 cm path length were used. The range scanned was 200–800 nm and 10 mM phosphate buffer (pH = 7.0) having an ionic strength (NaCl) of 50 mM was used as blank sample. The DNA sample was prepared by dilution with phosphate buffer (pH = 7.0) at room temperature. The concentration of the DNA stock was calculated from A_{260} (molar extinction coefficient, $\epsilon = 6600\ M^{-1}\ cm^{-1}$) per nucleotide phosphate. BSA stock solution was prepared in phosphate buffer (10 mM). For titration experiments the required aliquots of samples were added just prior to the experiment and mixed thoroughly. The stock solutions and samples were always protected from external light sources. For titration in the presence of CT DNA, the platinum concentration was kept constant at 50 μM and aliquots from the concentrated DNA solution were added gradually at different ratios. The *R* values were varied from 0 to 10, where *R* represented the ratio of DNA concentration in nucleotide phosphate per metal.

2.3. Circular dichroism

Circular dichroism spectra were recorded at 37 °C using a Jasco J-815 Spectropolarimeter equipped with a Jasco PTC-423 S Peltier temperature controller. The used scanning rate was 100 nm min^{-1}

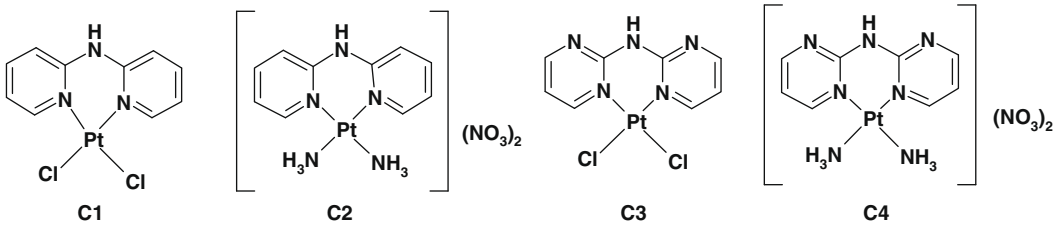


Fig. 1. Molecular structures of the four platinum compounds: $[Pt(dpa)Cl_2]$ **(C1),** $[Pt(dpa)(NH_3)_2](NO_3)_2$ **(C2),** $[Pt(dipm)Cl_2]$ **(C3)** and $[Pt(dipm)(NH_3)_2](NO_3)_2$ **(C4)**.

with a response time of 1 s. Spectra were recorded at standard sensitivity (100 mdeg) with a data-pitch of 0.5 nm in the continuous mode. The scanning range was 320–220 nm (for DNA) or 300–180 nm (for BSA). All the spectra are the average of four consequent accumulations. The cuvettes used were Hellma precision cells made of Quartz Suprasil of 2 mm path length. The baseline was corrected using a reference of 10 mM PBS. The sample volume in the cuvettes was kept fixed at 700 μL.

A typical sample containing DNA/protein and metal compound in phosphate buffer was incubated at 37 °C and the spectra were collected at the given time intervals. In addition the free ligands were also investigated under similar conditions. As reference compounds, cisplatin and ethidium bromide (EthBr) were also followed for similar time- and concentration-dependent experiments.

2.4. DNA binding studies

Supercoiled φX174 phage DNA (20 μM base pairs end concentration) was incubated with varying concentrations of the relevant compounds in 10 mM phosphate buffer (pH = 7). All reactions contained a compound:$CuSO_4$:ascorbic acid ratio of 4:1:16, unless otherwise noted. Ascorbic acid and $CuSO_4$ together (without added compound) result in unfolding of the DNA and therefore this mixture was used as control for all experiments. The compounds alone do not cleave when incubated with DNA. Samples were incubated in the dark at 37 °C for 30 min. After incubation, the reaction was quenched by the addition of DNA loading buffer containing 1 mM of the Cu(I) chelator BCDA (bathocuproine disulfonic acid). Samples were run on a 1% agarose gel in TAE buffer, stained with EthBr, and imaged with a BioRad ChemiDoc XRS apparatus.

2.5. Partition coefficient measurement

The partition coefficient was determined by a typical water/*n*-octanol biphasic partition method. The samples were prepared at a concentration of 50 μM in 0.15 M saline by weighing out the appropriate amount of the platinum compounds and adding the solvents. These conditions were found sufficient to prevent rapid hydrolysis of **C1** and **C3**. In all cases 3 mL of saline solution was mixed with 3 mL of saline saturated *n*-octanol. This biphasic solution mixture was shaken overnight by an automatic shaker and later centrifuged at 13,000 rpm for 15 min. Then 1 mL aliquots of both layers (top layer: *n*-octanol and bottom layer: aqueous) were collected separately. The aqueous sample was diluted 20-fold and the total platinum content was determined by ICP-OES (inductively coupled plasma–optical emission spectroscopy). The *n*-octanol samples were injected to a FAAS and the total platinum content was determined. Each experiment was duplicated and results have been averaged.

The platinum concentration of samples in octanol was determined using a graphite-oven flameless atomic absorption spectroscope (FAAS), Perkin–Elmer 3100 AAS apparatus, equipped with a platinum hollow cathode lamp and an AS-60 graphite-oven autosampler. All the measurements were repeated in triplicate and for each measurement 20 μL of sample was injected directly. The furnace was purged with argon gas and before starting a new sample the equipment was washed thoroughly with 1% HNO_3 solution. The furnace was programmed as mentioned below: drying 200 °C for 100 s, ashing 1300 °C for 60 s (first cycle) and 20 °C for 15 s (second cycle), atomization and measurement 2650 °C/5 s, purging 2600 °C/5 s. The wavelength of the lamp is 265.9 nm with a slit width of 0.70 nm.

The platinum concentration of samples in the aqueous phase was determined by ICP-OES. Measurements were carried out using a VISTA-MPX CCD Simultaneous ICP-OES instrument equipped with a Varian SPS 3 auto sampler. The samples were diluted 20-fold using a 3% HNO_3 solution and 3 mL of sample was injected. The wavelength used was 214.424 nm and each measurement was performed in duplicate. The average values of both phase measurements have been calculated for the final log *P* values.

3. Results and discussion

3.1. General comments

In the present study four platinum compounds (**C1–C4**) are studied for their mode of action, which have several differences in structures and properties (both chemically and electronically), with the aim to investigate the bio-physical properties and *in vitro* cytostatic activities. The changes in the ancillary ligands induce modifications of the chemical properties of the Pt complexes which should affect their interaction with different biological targets. These results are useful to correlate the bio-physical studies with *in vitro* cytotoxicity data.

3.2. UV–vis titration with Calf Thymus DNA

Electronic spectroscopy in the UV–vis range allows studying the interaction of metal compounds with bio-molecules, like DNA or proteins. The absorption spectra of the four platinum compounds in the presence of increasing amounts of DNA, with 50 μM Pt-compound are shown in Fig. 3. Compounds **C1** and **C2** show similar behavior on absorption spectral titration as both of them are coordinated to a common ligand, **dpa**. The chloride and ammine ligands induce small differences in the spectra at high wavelength absorption. There are four peaks observed at 252, 278, 306 and 336 nm for **C1** and at 204, 242, 301 and 333 nm for **C2,** respectively. Both compounds show a gradual increase in intensity against a blank DNA, which agrees with coordination binding to DNA. A subtle difference in their mode of binding is possible as **C1** can bind to DNA in either mono- or bidentate fashion and **C2** binds to DNA only by electrostatic and partial intercalation.

The UV–vis spectra of **C3** and **C4** are also similar in appearance, as both of them contain the ligand **dipm** with only a very small difference in spectra at a wavelength around 300 nm. There is a prominent peak for **C3** at 257 nm and a shoulder at 316 nm, whereas for **C4** the prominent peak appears at 258 nm with a shoulder at 318 nm and a very weak peak at 365 nm (which is not present for **C3**). For both **C3** and **C4**, there is no prominent change in intensity of the 316 nm peak, though a significant increase in intensity at 257 nm peak is noteworthy. For a specific *R* (*R* = 1), there is an insignificant blue shift (2 nm) (from 257 to 255 nm for **C3** and from 258 to 256 nm for **C4**). The hyperchromism exhibits the typical evidence of strong DNA binding, but the relatively small differences in spectra indicate only a slight variation in the mode of interaction. Therefore, the possible binding for **C3** might be coordinative binding, whereas for **C4** it could be electrostatic binding to the phosphate backbone, with also partial intercalation.

3.3. Circular dichroism with calf thymus DNA

The difference in absorption of polarized light gives rise to a CD spectrum, which can distinguish subtle changes in DNA conformational changes in solution. In biological systems the most abundant form of DNA present is the "B-form", which gives two characteristic peaks in the scan range of 220–320 nm [36]. Upon interaction of metallodrugs with DNA, there are several possibilities of non-coordinative binding modes [3]. The platinum antitumour compounds are known to be DNA-targeting drugs, so the interaction with DNA might be helpful to predict their mode of action. Different platinum

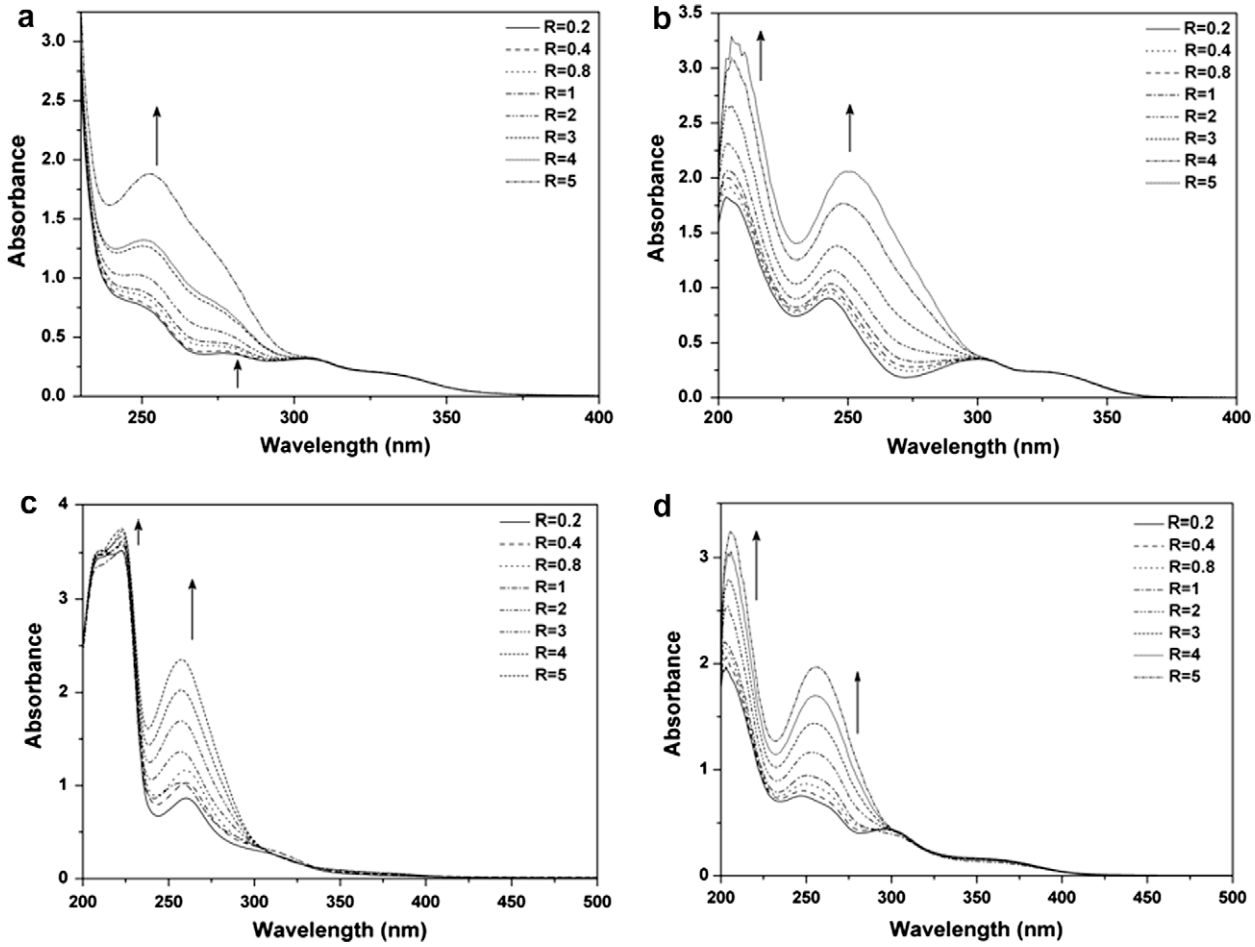


Fig. 3. UV–vis spectral changes of (a) **C1**, (b) **C2**, (c) **C3** and (d) **C4** on addition of CT DNA. Arrows denote the gradual changes on increasing amounts of DNA (10–250 μM) in the presence of a constant concentration (50 μM) of platinum compound at room temperature.

drugs react with DNA at different modes of binding [37], so CD studies may support the prediction of the mode of interaction.

The CD spectra were recorded at a constant DNA concentration of 100 μM and different molar ratios of the metal compounds (10–1000 μM). The value of *R* is defined as the concentration ratio of DNA to metal compound (or ligands), which varied from 10 to 0.1 depending on the concentration of platinum compounds or the ligands. After the measurements at time zero, incubation at 37 °C over a 24 h time period and measurements at several intervals have been performed. A long incubation time was allowed to favor sufficient time span for the slow activation *via* hydrolysis for **C1** and **C3**.

All the four platinum compounds exhibit significant changes in the CD bands, when compared to free DNA of same concentration. The spectral changes of the CT DNA in the CD spectra are shown in Figs. 4 and 5 with different experimental conditions. **C1** and **C3** show similar changing patterns, whereas **C2** and **C4** induce similar changes in CD signals. For **C1** and **C3** the negative band shows more noticeable changes than the positive peak at 275 nm. For **C3**, two smaller new peaks arise at around at 250 and 290 nm, whereas the negative band shows 3 nm of a red shift (bathochromic shift) with a sharp signal. The increase in the negative band is gradual with increasing amounts of the platinum compound. Thus, the base stacking is little disturbed by interaction with **C1** and **C3**, whereas the right-handed helicity of B-DNA has started to change. The differences observed between the compounds **C1** and **C3** might be explained by the influence of the coordinated diimine ligands.

The CD spectra of CT DNA in the presence of compounds **C2** and **C4** show similar changes in the positive band, but different changes in the negative band. The subsequent decrease in the positive bands with increasing compound concentration shows the instability of the base stacking and at the highest compound concentration; the B-DNA canonical form is almost lost. For **C4**, the intensity of negative band starts decreasing and also the sharp peak at 215 nm starts disappearing. This compound shows no apparent changes in base stacking, whereas at the highest concentration of the compound (ten times higher compared to the DNA concentration), the appearance of a new positive ellipticity at 230 nm is significant. The negative band shows a gradual red shift up to 6 nm.

For **C2**, though the B-DNA form is retained (except for highest concentration of compound), there is no increase in the positive or negative bands (induced by classical intercalation). **C2** and **C4** are cationic compounds with no labile leaving group, so the coordination core around platinum is expected to remain unchanged. The dipositive charge of the compound initially facilitates electrostatic attraction between the compound and the negatively charged DNA helix. The ligands around the platinum centers can form effective H-bonds, so they either can stabilize the DNA conformation, or may self-aggregate parallel to DNA helix. The highest concentration of the compound may easily aggregate to form a helical structure along the DNA helix. In addition, the bidentate aromatic ligands, **dipm** and **dpa** both can lead the compounds to partial intercalation. So, a combined effect of both electrostatic interaction and partial intercalation may be a possible origin of the observed changes.

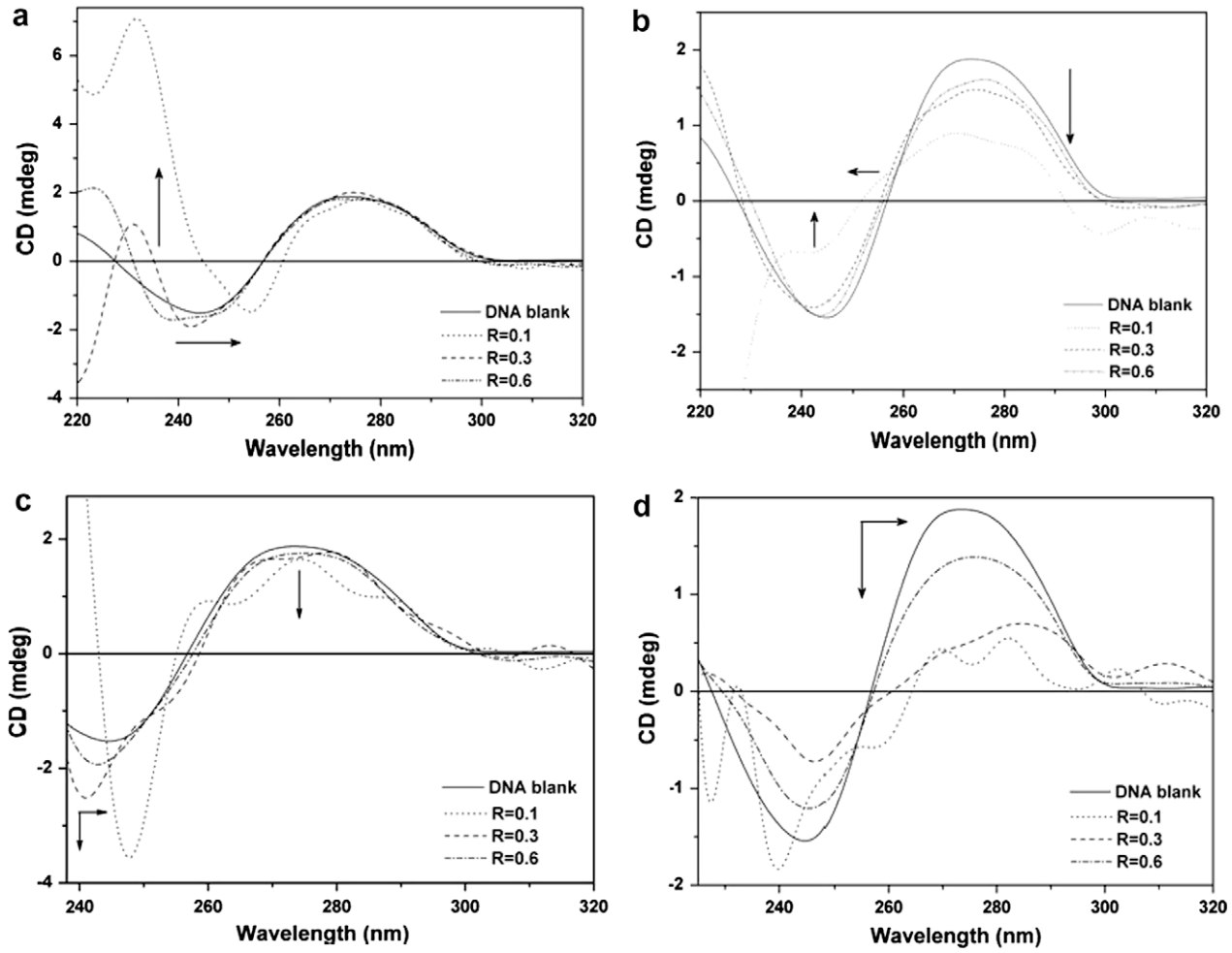


Fig. 4. CD spectral changes of CT DNA (100 μM) in phosphate buffered saline in the absence (–) and in the presence of (- - -, ····, - - -) increasing concentrations of (a) **C1**, (b) **C2**, (c) **C3** and (d) **C4**.

When the concentration of the platinum compounds is gradually reduced from 100 μM to 10 μM, while keeping the DNA concentration constant at 100 μM, the trend in CD spectral changes are less dramatic. For **C1** and **C3** the positive band (275 nm) was found to increase parallel with the appearance of a new ellipticity at 230 nm (**C3**) or at 225 nm (**C1**). The negative band for **C3** first increased and then gradually decreased and red-shifted for 2 nm. **C3** enhances the π-stacking, but the helicity of native DNA is inconclusive, as the changes in the negative band do not follow a typical trend. The changes in the spectra may be explained as a combined result of coordinative binding and partial intercalation. However, for **C1** the negative band keeps increasing, suggesting ongoing intercalation in the DNA.

For **C2** and **C4**, gradual increases in positive and negative bands are showing clearly the intercalative binding mode. For these two compounds, no new peak appears in the 225–230 nm range. Thus these two compounds show typical intercalation by enhanced π-stacking and stabilizing the right-handed helical structure of B-DNA.

The time-dependent spectra have also been measured for all these compounds. A significant time-effect on the changes of spectral band is observed. The observed spectral patterns are shown in the supporting information (Figs. S1–S4).

Several binding modes are possible for all these four platinum compounds (**C1–C4**), which also exhibit time-dependent intensity changes with shifts in wavelength. For all compounds, the higher concentration of platinum in the reaction solution induces conformational changes. The CD spectra of all four compounds maintained the conservative doublets with subtle, or almost no changes for $R = 1$. The presence of excess DNA in the titration solution induces hyperchromicity and bathochromic shifts (2–3 nm) for all the compounds, which is enhanced with longer incubation times. Therefore, the mode of interaction cannot be solely intercalation, but rather a combined effect of partial intercalation with coordinative binding and external stacking interactions. The optimized structures of all four platinum compounds depict the puckered form as shown in the preceding paper. Thus, the complete insertion through base pairs might not be facilitated. The dipositive charge on compound **C2** and **C4** assists the external electrostatic interaction, whereas for compounds **C1** and **C3**, coordinative binding is the other possible mode of interaction.

3.4. Interaction of platinum compounds with bovine serum albumin (BSA)

A major route for deactivation of platinum drugs is the stable adduct formation with serum proteins. Platinum compounds have a high affinity towards S-donor ligands (such as glutathione or metallothionein), so these adducts take a major part in bio-distribution, transport and removal of the active drugs. Therefore, the interaction of the present platinum compounds with albumin has been followed by UV–vis and CD spectroscopy.

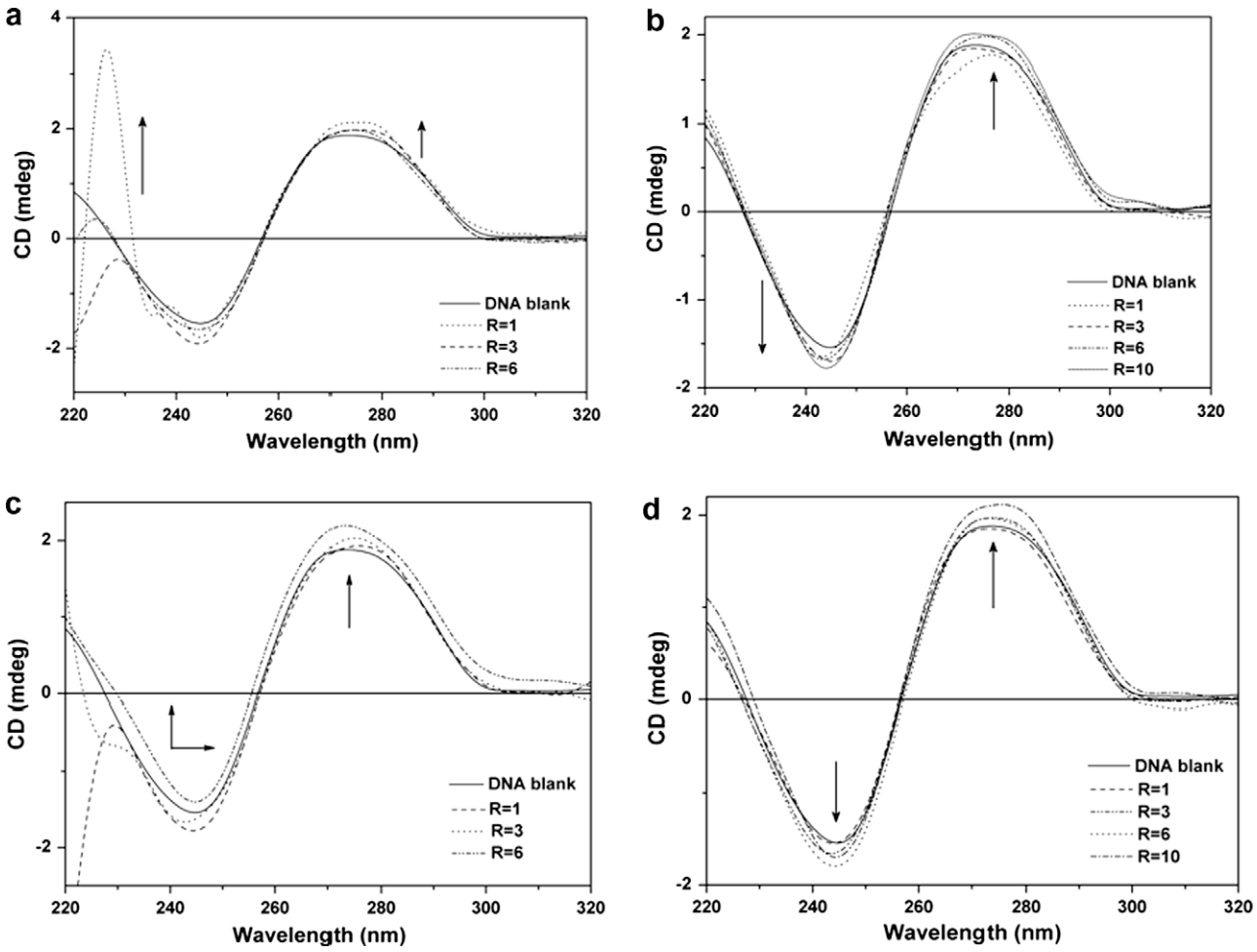


Fig. 5. CD spectral changes of CT DNA (100 μM) in phosphate buffered saline in absence (–) and in the presence of (- - -, ····, - - -) decreasing concentration of (a) **C1**, (b) **C2**, (c) **C3** and (d) **C4**.

In the absorption spectral titration, 10 μM BSA was used and different amounts of platinum compounds (concentrations varied from 1 μM to 100 μM) were added as described above for the DNA binding. This was followed by a thorough mixing before recording the spectrum. Five different ratios of BSA:platinum compound (R = 0.1, 0.5, 1, 5 and 10) were compared with the native BSA spectrum at room temperature at pH = 7.2 (no platinum compound present and the BSA concentration is 10 μM). The spectral changes are dramatic with higher concentration of the compound. For **C1** and **C2,** the three set of peaks appear at 279, 305, 336 (shoulder) nm and 285, 303, 330 nm, respectively. Hypochromism is observed when the Pt concentration is decreased gradually.

Three peaks are appearing at 261, 286 (shoulder), 314 nm for **C3** and at 265, 285 (shoulder) and 307 (shoulder) nm for **C4**. These peaks gradually increase with increasing concentration of the platinum compounds. When R is 1–10 (R is concentration ratio of protein to metal compound), the spectra return to the shape of native BSA with hypochromism for the peak at 279 nm (see Fig. 6).

As a reference compound, cisplatin was also tested for binding to BSA (Fig. S6). The known preferred binding site of cisplatin on BSA is methionine-298, but multiple binding at cysteine and tyrosine residues has also been reported [38,39]. After prolonged incubation 20 mol of platinum was found to bind to 1 mol of protein. The four novel platinum compounds thus agree with the binding mode of cisplatin as reported in the literature [27,40]. Therefore, a higher concentration of the platinum compound in the solution induces hyperchromicity indicating stronger binding. Conversely, hypochromicity is evident with lower platinum concentrations [40].

The CD studies thus provide novel insight in the changes in the secondary structure of BSA upon platinum binding. To correlate with the interaction mode of cisplatin (a classical coordinative binder) under same experimental conditions, changes in the CD were recorded after incubation with BSA (Fig. 7). The most abundant serum protein, serum albumin, (52% with 40–45 g per liter content for healthy humans) at physiological pH remains predominantly in the α-helical form (67%). The typical characteristic of this α-helix is evident in the CD spectra for the 180–300 nm scanning range. The two strong negative bands in the CD spectrum of bovine serum albumin appearing at 208 and 222 nm originate from π–π^* and n–π^* exciton transition. The investigated compounds do not give rise to any signal in this range. Thus, following the change in the ellipticity of these two bands, the extent of secondary structure change in protein can be readily assessed.

Increased concentrations of metal compounds (5, 50 and 500 μM, respectively) to a solution of the protein (50 μM) were used. The cisplatin analogues, **C1** and **C3**, exhibit a similar behavior in the CD spectra as cisplatin. The absorption maximum of 222 nm shows a red shift of 8 nm in the presence of a 10-fold excess of the platinum compound (R = 0.1, where R stands for the concentration ratio of protein per platinum compound). In addition, the ellipticity at 208 nm completely disappeared with increasing amount of compound. The common mode of interaction of cisplatin with BSA is the coordinative binding after hydrolysis of chloride ions. These

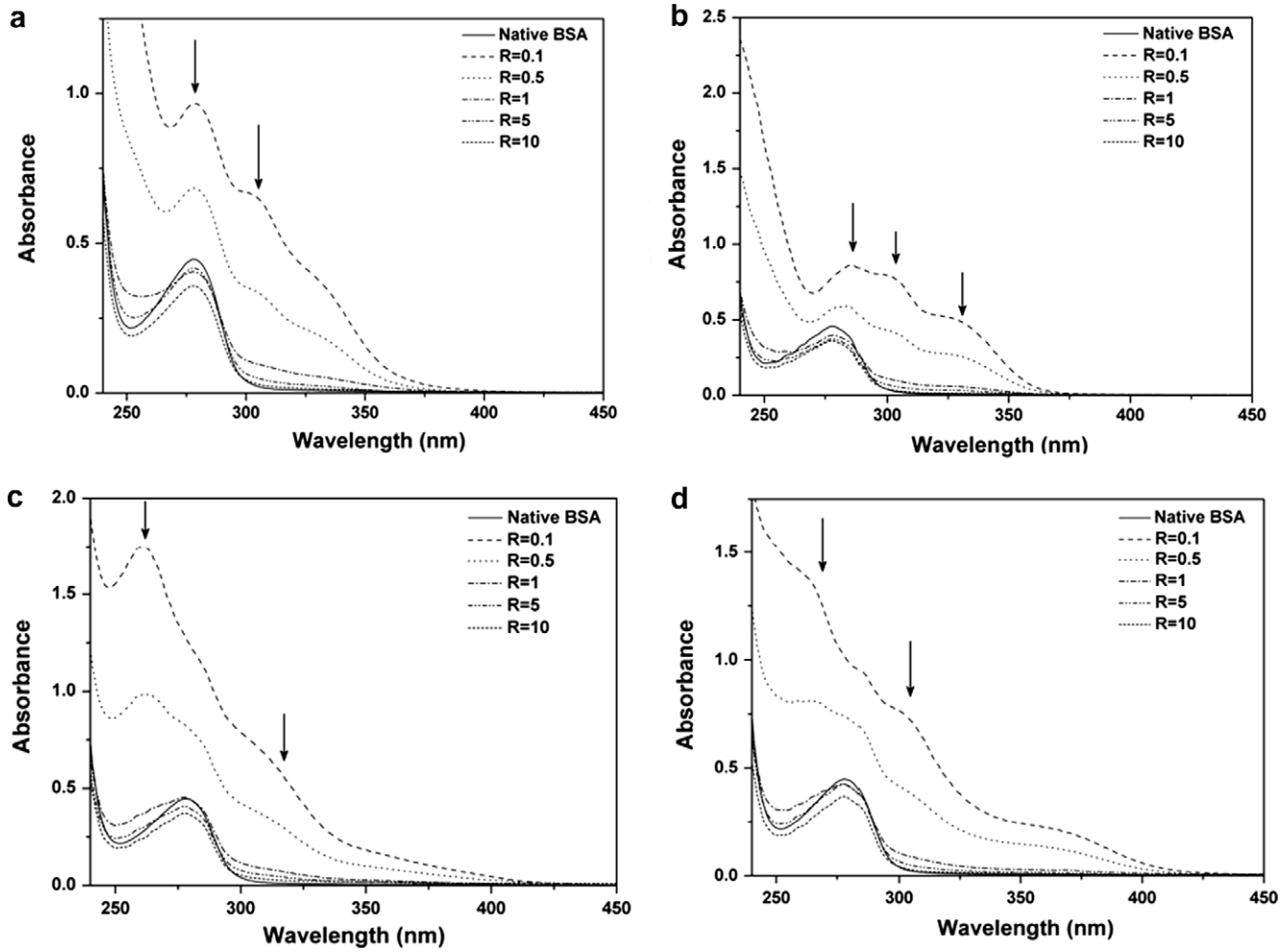


Fig. 6. Changes in the UV–vis spectra after incubation of a constant concentration of protein (50 μM) with different concentration of (a) **C1**, (b) **C2**, (c) **C3** and (d) **C4** (500–5 μM) at pH 7.2 in phosphate buffered saline at room temperature. Arrows show the changes in absorption upon reduction of platinum compounds and *R* is the concentration ratio of protein to platinum compound.

two new platinum compounds react in a similar fashion as cisplatin. After hydrolysis of one chloride ligand, a positively charged intermediate is formed. This highly reactive intermediate binds to BSA and destabilizes the native α-helical form. The destabilization is clearly evident from the CD spectra.

However, **C2** and **C4** contain non-hydrolysable ammine ligands. Therefore, upon addition of increasing amounts of each compound, very few changes are observed. The stable helical form is retained with only a little increase in ellipticity, which can be explained by external attachment of the platinum to the protein.

3.5. Partition coefficient measurement

In order to measure the selective preference of a solute, the distribution property was measured by the shake-flask method. The two immiscible solvents chosen are water and octanol (octanol mimics the lipid membrane, or cell wall [41]). The four platinum compounds (**C1–C4**) along with cisplatin are used to determine the partition coefficient. Only **C1** exhibits a positive log $P_{o/w}$ value indicating a very high lipophilicity. The other log $P_{o/w}$ values are negative, showing high hydrophilicity. **C3** shows a partition coefficient comparable with satraplatin. **C2** and **C4** show partition coefficients comparable to oxaliplatin. If these compounds are arranged according to the order of increasing lipophilicity the trend will be **C1** > **C3** > **C2** > **C4**. The comparison of the distribution with few others reported platinum drugs are summarized in Table 1.

3.6. DNA binding and cleavage studies by gel electrophoresis

DNA binding or cleavage by compounds was analyzed by determining the relative migration rates of the different DNA isoforms during agarose gel electrophoresis. The native DNA remains in the supercoiled form, also known as covalently coiled coil (CCC) DNA, here designated Form I. Single-strand cleavage results in so-called nicked or open circular DNA (designated Form II), whereas the double-strand cleavage results in linear DNA (designated Form III). The migration rate during agarose gel electrophoresis depends on both size (base pairs) and conformation, with smaller and/or condensed DNA migrating faster than larger and/or unfolded DNA. Supercoiled DNA (Form I) has a tightly packed conformation and therefore migrates faster through agarose gels than linear DNA (intermediate migration) or open circle DNA (slowest migration). In our experiments, we always used the same DNA (*i.e.*, same size) and therefore the migration rate depended primarily on the conformation of the DNA, changes of which were induced by the various platinum compounds. In fact the non-linear increased cleavage with increasing compound concentration suggests cleavage at multiple sites by the platinum compound.

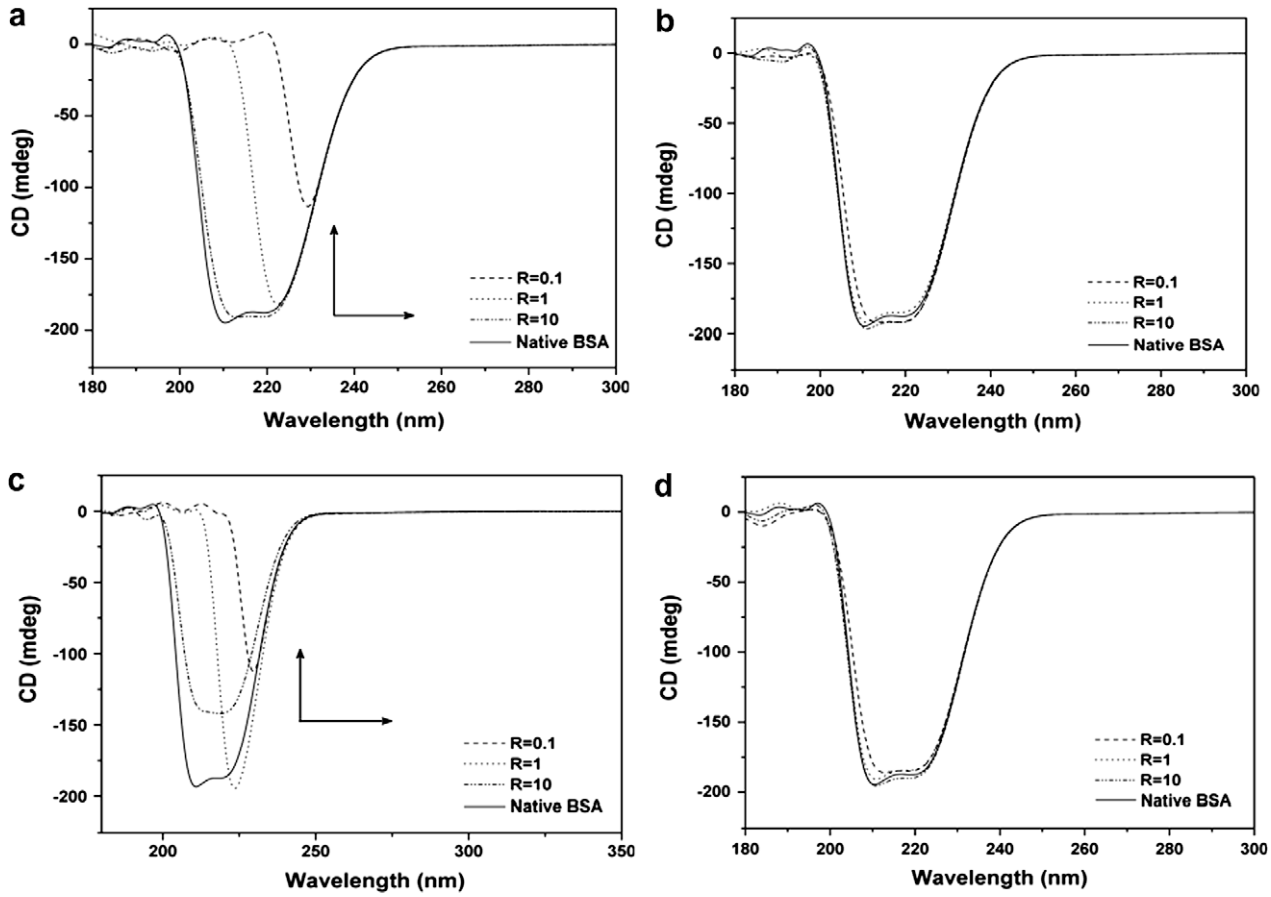
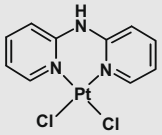
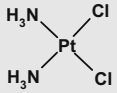
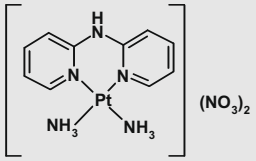
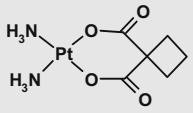
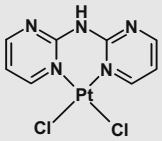
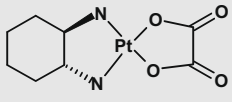
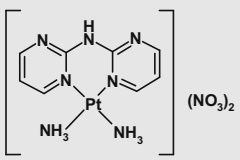
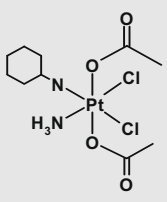


Fig. 7. Changes in CD spectra after incubation of a constant concentration of protein (50 μM) with different concentration of (a) **C1**, (b) **C2**, (c) **C3** and (d) **C4** (500–5 μM) at pH 7.2 in phosphate buffered saline at room temperature.

Table 1
Comparison of partition coefficient values for several reported platinum compounds using known methodology in water/*n*-octanol [31–33].

Studied compounds	Structure	log *P*	Reported compounds	Structure	log *P*
C1, [Pt(dpa)Cl_2]		0.11	Cisplatin		−2.53
C2, [Pt(dpa)$(NH_3)_2$]$(NO_3)_2$		−1.33	Carboplatin		−2.3
C3, [Pt(dipm)Cl_2]		−0.47	Oxaliplatin		−1.65
C4, [Pt(dipm)$(NH_3)_2$]$(NO_3)_2$		−1.74	Satraplatin		−0.16

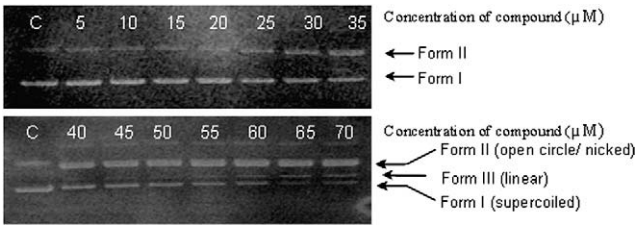


Fig. 8. Agarose gel electrophoresis of φX174 DNA after 30 min incubation with **C3**. Top gel, 5–35 μM; bottom gel, 40–70 μM. Form II appears at around 35 μM, when almost all Form I has been converted to Form II. This suggests two separate single-strand cleavage events. Also, at 55 μM, the DNA is it still primarily in Form II, with only a little Form III appearing. Also note that the supercoiled DNA migrates slower at higher compound concentration, most likely as the result of increased adduct formation.

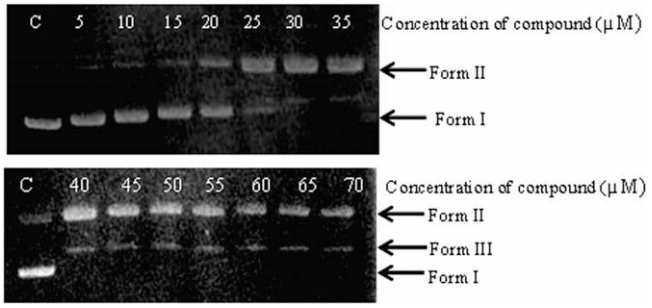


Fig. 9. Agarose gel electrophoresis of φX174 phage DNA after 30 min incubation with **C4**. For explanation see Fig. 8. **C4** cleaves DNA in a similar fashion as **C3**, but significantly more efficiently (all supercoiled DNA is converted to open circle DNA at 30 μM for **C4**, while at 70 μM **C4** Form I can still be observed).

Two platinum compounds (**C3** and **C4**) were tested for their DNA cleavage properties, using supercoiled φX174 phage DNA in phosphate buffer at 37 °C. A typical sample contained 20 μM DNA (in base pairs) in the absence or in the presence of increasing amount of compounds or reducing agents. All the samples were incubated for 0.5 h with exclusion of light.

C3 led to DNA cleavage, whereby Form I decreased and was gradually replaced by Form II (appearance started at 35 μM resulting from single-strand cleavage) and finally also Form III started appearing at 55 μM (Fig. 8). In these experiments it is clear that two-step single-strand cleavage is taking place. In other words the cleavage process took place via the route: first covalently coiled coil (CCC) to open circular (OC) and then open circular (OC) to linear. Interestingly, the band corresponding to supercoiled DNA migrated slower at increasing compound concentration. This is best explained by the simultaneous and strong binding to DNA of multiple **C3** molecules, resulting in a significant change in the molecular mass and conformation of the DNA.

On the other hand, **C4** shows strong binding from very low concentration (5 μM), as evidenced by retardation in Form I migration shift in the gel. The appearance of Form III clearly starts from 35 μM concentration (Fig. 9). **C4** is a significantly better cleaving agent than **C3**. The amount of linear DNA (Form III) produced by **C4** at 30–35 μM requires at least 70 μM **C3**. Furthermore, while all supercoiled DNA (Form I) is cleaved by 30 μM **C4**, at 70 μM **C3** still some supercoiled DNA is observed.

The effect of reducing agent (Ascorbic Acid, AA) on the cleavage of **C4** was also assessed (Fig. 10). **C4** exhibits 50% single-stranded cleavage stoichiometrically after 30 min of incubation (Pt to DNA = 1.25:1). The cleavage was much stronger in the presence of ascorbic acid, as evidenced by the stronger increase in Form II (open circular) DNA relative to the reactions performed in the absence of reductant. In the absence of ascorbic acid, the cleavage was negligible, even when Pt compound was in excess (1:2.5 ratio of DNA: Pt). However, it is important to note that the reduced mobility of Form I with increasing amounts of the platinum compound was found to be independent of the presence of ascorbic acid, strongly suggesting that only the cleavage and not the DNA binding depends on the reductant. This observation further supports the notion that the reduced migration rate of the supercoiled DNA is due to binding and unfolding and not to cleavage of the DNA by the compounds **C3** and **C4**.

4. Conclusions

Several platinum compounds have been investigated for their mode of interaction with two major biological targets. The neutral cisplatin-analogues show coordinative binding and partial intercalation. The interaction with BSA further indicates a similar activation pathway (i.e. *via* hydrolysis of chloride ligand) as cisplatin. However, the cationic platinum compounds appear to have an electrostatic interaction with the negative DNA-phosphate backbone, followed by partial intercalation. The ammine ligands on these compounds further stabilize the DNA adduct by hydrogen bonding. The other possible modes of interaction with DNA are external association with or without self-aggregation. The non-planar folded structures of all these compounds inhibit the complete intercalation. The gel-mobility studies additionally clarify multiple binding to and subsequent reductant-dependent cleavage of DNA strands by the platinum compounds. The reaction mode is two consecutive single-strand cleavages. From the electrophoresis studies it is clearly evident that a gradual formation of From III via From II is taking place. In the absence of ascorbic acid adduct formation of compounds **C3** and **C4** to the DNA was not impaired, but cleavage was not observed.

A high affinity towards serum albumin is exhibited by neutral cisplatin analogues. The stable α-helical conformation of the protein (BSA) is destabilized by these compounds whereas the cationic compounds do not induce any significant change of the BSA conformation in the CD spectral time scale. Therefore, the probability of retaining the intact formulation in cellular milieu is higher for cationic compounds. The neutral compounds may therefore be deactivated or removed by BSA–platinum drug adduct formation. This selective affinity towards platinophiles directs a synthetic design of cationic compounds with higher activity *in vivo*.

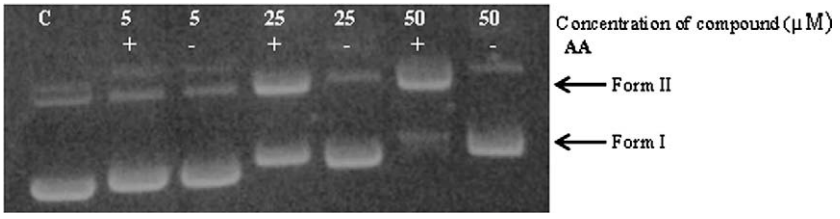


Fig. 10. Agarose gel electrophoresis of φX174 phage DNA after 30 min incubation with **C4**, in the presence (+) or absence (−) of ascorbic acid at a concentration of 5, 25 or 50 μM.

The partition coefficients for platinum compounds are in a comparable range with clinically approved drugs, except **C1** [Pt(dpa)Cl_2]. This compound is clearly lipophilic and thus will be facilitated significantly to cross the cellular membrane. The other three compounds are hydrophilic and are more likely to accumulate in the blood plasma. Both cationic compounds show lipophilicity in the range of oxaliplatin. Thus, lipophilicity tests can provide a rough idea of the behavior of a compound *in vivo*, but no straightforward correlation can be drawn between lipophilicity and *in vivo* anticancer activity.

In the preceding article (dealing with the synthesis, model-base studies, DFT calculation and *in vitro* growth inhibition studies against seven human tumor cells), it has been reported that the most active compound in these series is **C3**, Pt(dipm)Cl_2. The cationic compounds, **C2** and **C4**, however, exhibit selective activity against breast cancer cell line EVSA-T. In order to explain and correlate the low growth inhibition ability of these compounds, the bioavailability along with DNA and BSA interaction has been investigated. The three compounds (except **C1**) being lipophobic probably face lower transport to the cells and greater accumulation in the serum. The lipophilicity of **C1** should facilitate facile transport inside the cells, however; the strong proteinophilic behavior may limit the amount of active component for the DNA. The other neutral compound, **C3,** in spite of its hydrophilic property, can cross the cellular membrane and induce the growth inhibition. The cationic compounds being hydrophilic would also have slower affinity to cross the cell-membrane. These compounds (**C2** and **C4**) have additional ammine ligands which would enhance the DNA adduct. This assumption is proved by electrophoresis studies. The limited activity against the cancer cells thus could be reasoned by reversible DNA damage and easily reparable cleavage products. All four compounds interact with purified DNA (a primary cellular target), but the cellular processing is yet to be investigated to explain the antitumor activity and to modify the ligand structure for a better antitumor active compound.

Acknowledgements

Johnson-Matthey (Reading, UK) is gratefully thanked for their generous loan scheme of K_2PtCl_4. The support and sponsorship provided by COST Actions D39/0006/02 and D39/0005/11, allowing exchange of preliminary results and discussion, is sincerely acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2009.07.003.

References

[1] W.C. Johnson, in: K. Nakanishi, N. Berova, R.W. Woody (Eds.), Circular Dichroism: Principles and Applications, VCH Publishers Inc., New York, 1994.
[2] G. Snatzke, Circular Dichroism: Principles and Applications, VCH, 1994.
[3] A.D. Richards, A. Rodger, Chem. Soc. Rev. 36 (2007) 471–483.
[4] J.A. Rice, D.M. Crothers, A.L. Pinto, S.J. Lippard, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 4158–4161.
[5] J.M. Teuben, C. Bauer, A.H.J. Wang, J. Reedijk, Biochemistry 38 (1999) 12305–12312.
[6] A.L. Pinto, S.J. Lippard, Biochim. Biophys. Acta 780 (1984) 167–180.
[7] J.J. Roberts, A.J. Thomson, Prog. Nucleic Acid Res. Mol. Biol. 22 (1979) 71–133.
[8] J. Reedijk, A.M.J. Fichtinger-Schepman, J.H.J. Denhartog, Inorg. Chim. Acta 79 (1983) 252–253.
[9] E.D. Scheeff, J.M. Briggs, S.B. Howell, Mol. Pharmacol. 56 (1999) 633–643.
[10] S.E. Sherman, D. Gibson, A.H.J. Wang, S.J. Lippard, Science 230 (1985) 412–417.
[11] S.E. Sherman, D. Gibson, A.H.J. Wang, S.J. Lippard, J. Am. Chem. Soc. 110 (1988) 7368–7381.
[12] S.E. Sherman, S.J. Lippard, Chem. Rev. 87 (1987) 1153–1181.
[13] E.G. Talman, W. Bruning, J. Reedijk, A.L. Spek, N. Veldman, Inorg. Chem. 36 (1997) 854–861.
[14] B.K. Teo, P. Eisenberger, J. Reed, J.K. Barton, S.J. Lippard, J. Am. Chem. Soc. 100 (1978) 3225–3227.
[15] C.J. Van Garderen, M.J. Bloemink, E. Richardson, J. Reedijk, J. Inorg. Biochem. 42 (1991) 199–205.
[16] Y.S. Wu, K.R. Koch, V.R. Abratt, H.H. Klump, Arch. Biochem. Biophys. 440 (2005) 28–37.
[17] D.Z. Yang, S. Van Boom, J. Reedijk, J.H. Van Boom, N. Farrell, A.H.J. Wang, Nat. Struct. Biol. 2 (1995) 577–586.
[18] G.M. Blackburn, M.J. Gait, Nucleic Acids in Chemistry and Biology, Oxford University Press, New York, 1996.
[19] M. Cusumano, M.L. Di Pietro, A. Giannetto, F. Nicolo, E. Rotondo, Inorg. Chem. 37 (1998) 563–568.
[20] D. Gibson, K.F. Gean, R. Benshoshan, A. Ramu, I. Ringel, J. Katzhendler, J. Med. Chem. 34 (1991) 414–420.
[21] K.W. Jennette, J.T. Gill, J.A. Sadownick, S.J. Lippard, J. Am. Chem. Soc. 98 (1976) 6159–6168.
[22] L.M. Chen, J. Liu, J.C. Chen, C.P. Tan, S. Shi, K.C. Zheng, L.N. Ji, J. Inorg. Biochem. 102 (2008) 330–341.
[23] S. Kashanian, M.B. Gholivand, F. Ahmadi, A. Taravati, A.H. Colagar, Spectrochim. Acta A 67 (2007) 472–478.
[24] S. Kemp, N.J. Wheate, D.P. Buck, M. Nikac, J.G. Collins, J.R. Aldrich-Wright, J. Inorg. Biochem. 101 (2007) 1049–1058.
[25] M. Cusumano, M.L. Di Pietro, A. Giannetto, P.A. Vainiglia, Eur. J. Inorg. Chem. (2005) 278–284.
[26] N.S. Quiming, R.B. Vergel, M.G. Nicolas, J.A. Villanueva, J. Health Sci. 51 (2005) 8–15.
[27] A.R. Timerbaev, C.G. Hartinger, S.S. Aleksenko, B.K. Keppler, Chem. Rev. 106 (2006) 2224–2248.
[28] M.J. Bloemink, J.M.J. Perez, R.J. Heetebrij, J. Reedijk, J. Biol. Inorg. Chem. 4 (1999) 554–567.
[29] S. Roy, J.A. Westmaas, F. Buda, J. Reedijk, J. Inorg. Biochem. 103 (2009) 1278–1287.
[30] J.A. Platts, S.P. Oldfield, M.M. Reif, A. Palmucci, E. Gabano, D. Osella, J. Inorg. Biochem. 100 (2006) 1199–1207.
[31] Y.A. Lee, Y.K. Chung, Y.S. Sohn, J. Inorg. Biochem. 68 (1997) 289–294.
[32] D. Screnci, M.J. McKeage, P. Galettis, T.W. Hambley, B.D. Palmer, B.C. Baguley, Brit. J. Cancer 82 (2000) 966–972.
[33] W.S. Ye, L.G. Lou, W.P. Liu, Y. Yu, X. Chen, S.Q. Hou, W.Q. Gao, Y. Liu, Bioorg. Med. Chem. Lett. 17 (2007) 2144–2147.
[34] W.B. Yao, K. Kavallieratos, S. de Gala, R.H. Crabtree, Inorg. Chim. Acta 311 (2000) 45–49.
[35] V.Y. Kukushkin, A. Oskarsson, L.I. Elding, N. Farrell, Inorg. Synth. 32 (1998) 141–144.
[36] V.I. Ivanov, L.E. Minchenk, A.K. Schyolki, A.I. Poletaye, Biopolymers 12 (1973) 89–110.
[37] J.P. Macquet, J.L. Butour, Eur. J. Biochem. 83 (1978) 375–387.
[38] A.I. Ivanov, J. Christodoulou, J.A. Parkinson, K.J. Barnham, A. Tucker, J. Woodrow, P.J. Sadler, J. Biol. Chem. 273 (1998) 14721–14730.
[39] J.F. Neault, H.A. Tajmir-Riahi, Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1384 (1998) 153–159.
[40] T. Peleg-Shulman, D. Gibson, J. Am. Chem. Soc. 123 (2001) 3171–3172.
[41] A. Ghezzi, M. Aceto, C. Cassino, E. Gabano, D. Osella, J. Inorg. Biochem. 98 (2004) 73–78.