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DNA binding of the anti-cancer platinum complex *trans*-[{Pt(NH_3)$_2$Cl}$_2$μ-dpzm]$^{2+}$ †

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The DNA binding of the dinuclear platinum complex *trans*-[{Pt(NH_3)$_2$Cl}$_2$μ-dpzm]$^{2+}$ (di-Pt), linked with the 4,4′-dipyrazolylmethane (dpzm) linker, was examined by ^{1}H and ^{195}Pt NMR and transcription assays. At 60 °C, di-Pt reacts with guanosine two-fold slower ($t_{1/2}$: 1 h) than cisplatin ($t_{1/2}$: 0.5 h). With adenosine, the di-Pt complex reacts much slower ($t_{1/2}$: 7 h) forming a range of different adducts through the N7 and either the N1 or N3 positions of the nucleoside. From ^{1}H NMR analysis of the major product of the reaction of di-Pt with the oligonucleotide d(ATGCAT)$_2$, purified by HPLC, it was determined that the dinuclear platinum complex can readily form a 1,2-GG interstrand DNA cross-link. Transcription assays using di-Pt and the *lac* UV5 promoter indicated that the metal complex forms an array of adducts vastly different from cisplatin. The two greatest blockages occurred at adenine residues, with possible interstrand and intrastrand AA and AG adducts being formed. These results indicate that unlike other platinum based anti-cancer agents, di-Pt binds to DNA with a preference for adenine bases.

Introduction

Since the initial discovery of the anti-cancer properties of cisplatin, and its subsequent world-wide approval for use in the clinic, thousands of other platinum based drugs have been made and tested for anti-cancer activity.[1] Potentially, the most important discovery has been the multi-nuclear platinum drugs, di- and trinuclear complexes that are linked by a variety of aliphatic and aromatic ligands.[2,3] Multi-nuclear platinum complexes show excellent activity both in cisplatin-sensitive and resistant cancer cell lines, with some being active at much lower concentrations than cisplatin.[2,3]

Multi-nuclear platinum complexes were initially synthesised on the basis that as they would form a different range of DNA adducts compared to cisplatin, they should therefore also display a different spectrum of anti-cancer activity to cisplatin.[2] Cisplatin binds at the N7 of both guanine and adenine bases, with the major adducts formed being (GpG) (47–50%) and (ApG) (23–28%) intrastrand DNA cross-links, with some interstrand DNA cross-links (<8%) observed.[4] This binding produces a rigid bend in DNA of 30–35° directed toward the major groove, and a localised unwinding of the DNA helix of around 13%.[4] Conversely, the DNA binding of multi-nuclear platinum complexes is generally characterised by the formation of long range intra- and interstrand cross-links at guanine residues.[2] These flexible, non-directional DNA adducts can induce DNA conformational changes from B-type to either A- or Z-type DNA.[2] However, despite considerable research into the mechanism of action of multi-nuclear platinum complexes, it is still not known which adducts are responsible for the excellent activity observed.[2,3]

It has been established that multi-nuclear platinum complexes will reversibly pre-associate in the DNA minor groove before forming covalent bonds with guanine residues in the major groove.[5–8] In a recent study, Qu *et al.* demonstrated that the terminal platinum centres of the trinuclear complex BBR3464 covalently bound at guanine N7 atoms in the major groove, while the inert central platinum centre resided in the minor groove.[9] This suggests that after the trinuclear platinum complex pre-associates in the minor groove the terminal platinum centres straddle the sugar phosphate backbone and bind to guanine N7 atoms in the major groove. Although multi-nuclear complexes will preferentially pre-associate in A/T rich regions of the minor groove,[5,6] there has been little evidence of covalent adducts being formed at adenine residues, either in the major or minor grooves. This is presumably due to the greater reactivity of guanine residues to electrophilic agents.

We have previously synthesised multi-nuclear platinum complexes linked by the 4,4′-dipyrazolylmethane (dpzm) ligand.[5,6,10] While the dpzm based complexes show good *in vitro* anti-cancer activity, and essentially maintain the activity in cisplatin resistant cell lines, they are not as active as the analogous aliphatic linked complexes (BBR3005 and BBR3464). As the preferential association of multi-nuclear platinum complexes at A/T rich regions in the minor groove is significantly stabilised by van der Waals forces, the heteroaromatic based dpzm complexes may exhibit a greater preference than the aliphatic linked BBR complexes. This, in turn, could lead to a greater probability of forming covalent bonds at adenine residues. While some adenine adducts have been reported for multi-nuclear platinum complexes,[11] no complexes to date have demonstrated a preference for adenine residues over guanine residues.

In this study we have examined the binding of the dinuclear platinum complex *trans*-[{Pt(NH_3)$_2$Cl}$_2$μ-dpzm]$^{2+}$ (di-Pt, Fig. 1) to DNA by means of a transcription assay that allows the determination of the nucleotide residues that the metal complex binds in a segment of DNA. In order to confirm the kinetic preference of our platinum complex for guanine residues, the binding of the di-Pt complex to the nucleotides adenosine and guanosine along with the oligonucleotide d(ATGCAT)$_2$ was also examined and compared to the results obtained with the transcription assay.

† Electronic supplementary information (ESI) available: The ^{195}Pt NMR spectra of di-Pt and di-Pt after reaction with guanosine and adenosine, the ^{1}H NMR of di-Pt after reaction with adenosine and an expansion of ^{1}H NOESY spectrum showing the aromatic H8/H6 to sugar H2′/H2″ region of the oligonucleotide d(ATG*CAT)$_2$ bound by di-Pt. See http://www.rsc.org/suppdata/dt/b3/b306417d/

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Table 1 The chemical shifts (ppm) of guanosine, metal complex and guanosine bound metal complex. ^{1}H NMR chemical shifts are reported at 60 °C, internally referenced to DSS (0 ppm). ^{195}Pt NMR resonances at 25 °C, are externally referenced to K_2PtCl_4 (−1631 ppm). Coupling (Hz) is shown in brackets

	^{1}H				
Metal complex/nucleoside	H8	H1′	H5	H3	^{195}Pt
Guanosine	8.01	5.93 (7.5)	···	···	···
Di-Pt	···	···	7.78	7.75	−2338
Cisplatin–gua	8.38	5.89 (6.5)	···	···	−2443
Di-Pt–gua	8.62	6.00 (6.5)	7.89	7.89	−2467

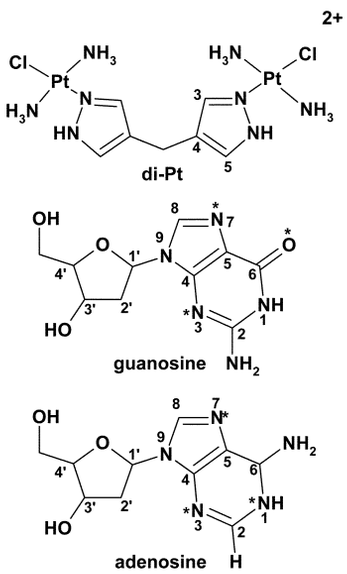

Fig. 1 Structure of the metal complex di-Pt and the nucleosides guanosine and adenosine, showing the possible binding sites (*) of platinum complexes and the numbering schemes used.

Results

Guanosine

Initially, attempts were made to monitor the reaction of di-Pt with 5′-GMP. However, on adding the metal complex to solutions containing 5′-GMP a white precipitate formed. The precipitate, which remained insoluble in hot water, contained both the 5′-GMP and di-Pt and probably occurred due to ion-pairing. As a result, it was decided to use the uncharged nucleoside, guanosine, to prevent precipitation from occurring.

The reaction of di-Pt with guanosine was followed by ^{1}H NMR over 24 h (Fig. 2). A large number of resonances are observed between 7.70 and 9.0 ppm, suggesting that the binding of the metal complex follows a step-wise reaction (Fig. 3). After 0.5 h, a decrease in the intensity of the guanosine H8 resonance is observed, while two new resonances are seen at 8.61 and 8.62 ppm consistent with platination at the N7 of guanine (see Table 1).[12–14] As the reaction proceeds the free guanosine H8 signal decreases until after 24 h it has completely disappeared. At the same time the resonance at 8.61 ppm also decreases in intensity while the resonance at 8.62 ppm becomes more intense. This indicates that the resonance at 8.61 ppm is from an intermediate product, presumably where only one guanosine is bound to the metal complex (complex II in Fig. 3). The resonance at 8.62 ppm (complex III) can then be assigned to the guanosine H8 from the di-substituted metal complex. During the course of the reaction a decrease in the intensity of the sugar H1′ (5.92 ppm) doublet is observed, as a new doublet at 6.00 ppm appears. After 24 h only the resonance at 6.00 ppm is observed with a coupling constant J = 6.5 Hz. No evidence of intermediate products is observed in the sugar H1′ region.

The resonances observed from di-Pt during the reaction (see Fig. 2) also confirm the step-wise reaction shown in Fig. 3.

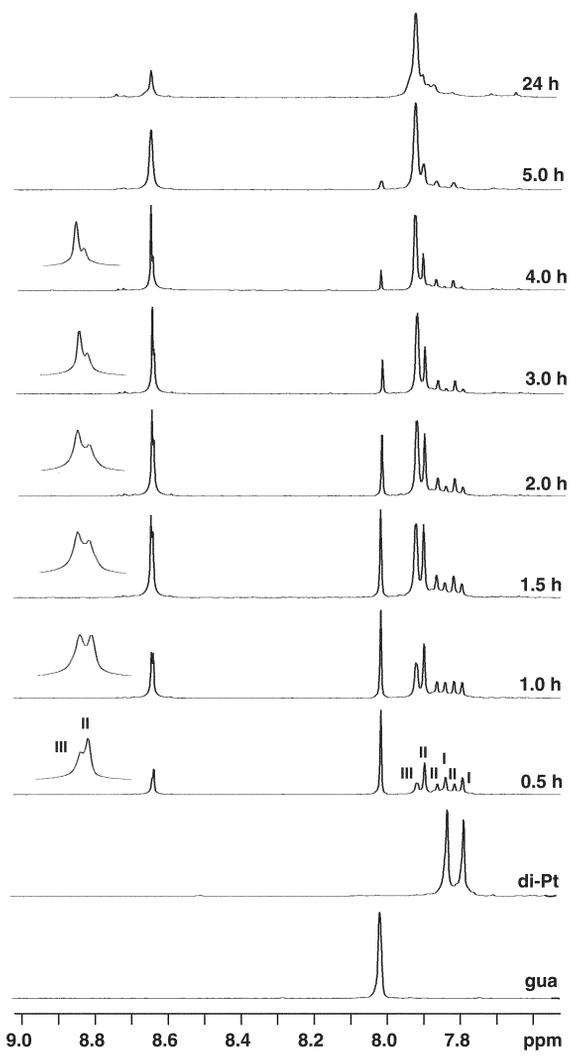

Fig. 2 The reaction of di-Pt with guanosine, at 60 °C, followed by ^{1}H NMR, over 24 h. Resonances for both the mono-substituted (II) and di-substituted di-Pt complex (III) are observed, consistent with the step-wise reaction shown in Fig. 3.

After 0.5 h, four new resonances are observed. The resonances at 7.84 and 7.79 ppm are from the H5/H3 protons of the unsubstituted end of the intermediate complex II, while the resonance at 7.87 ppm is from the H5/H3 protons of the bound end of the intermediate complex (based on a 2 to 1 integration). These resonances gradually decrease during the reaction until they are barely observed after 24 h. The fourth resonance at 7.89 ppm, which increases in intensity as the reaction proceeds is assigned as the coincidental resonances of the H5/H3 protons of the final di-substituted di-Pt complex (III). Analysis of the ^{195}Pt NMR spectrum of the di-Pt complex after reaction with guanosine at 60 °C for 24 h is consistent with the covalent binding of the metal complex to guanosine (see Fig. S1, ESI †).[12–14] After reaction the resonance at −2327 ppm has almost disappeared and a new resonance at −2467 ppm is observed indicating the formation of only one product.

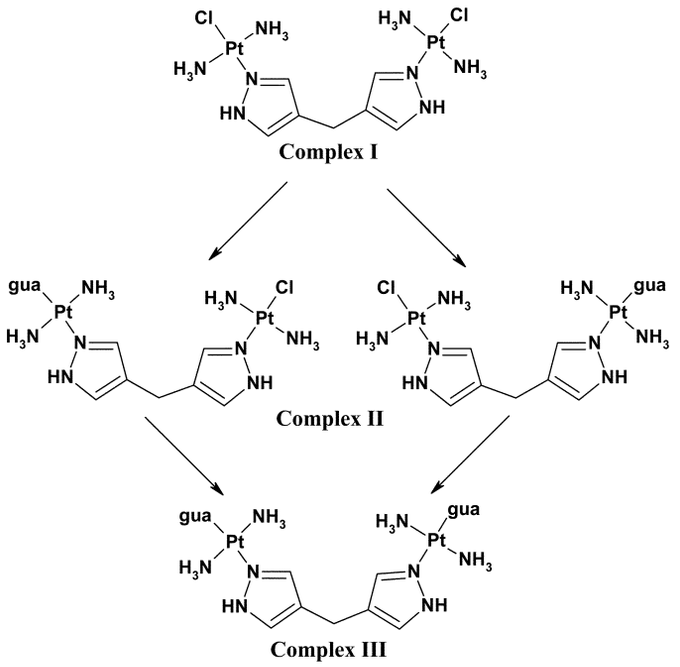

Fig. 3 A schematic diagram showing the step-wise substitution of both chloro ligands with guanosine. Peaks corresponding to complexes I, II and III are seen in the aromatic region of the ^{1}H NMR spectrum.

Adenosine

The reaction of di-Pt with adenosine was also followed at 60 °C by ^{1}H NMR. Unlike the reaction with guanosine, the ^{1}H NMR spectrum of di-Pt with adenosine indicates that di-Pt binds adenosine at a number of sites, including the expected N7 as well as at either the N1 or N3 (see Fig. S2, ESI †). Over a period of 24 h the resonances of the AH8 (8.34 ppm) and AH2 (8.28 ppm, assigned from T_1 experiments where the H8 resonances are positive and the AH2 resonances are negative at particular τ times) slowly disappear, with many new resonances appearing further downfield. New AH8 resonances are seen between 9.1 and 9.4 ppm and new AH2 resonances between 8.7 and 9.1 ppm (assigned from T_1 experiments). These downfield shifts (~0.6 to 1.0 ppm) for both protons are consistent with covalent binding by di-Pt at the N7 and N1/N3 sites of adenosine.[13] The results suggest that many different adducts are formed (*e.g.* N7–N7, N7–N3, N3–N1), unlike guanosine where only N7–N7 binding is observed. However, it was not possible to differentiate N1 binding from N3 binding. By comparing the percentage of the free guanosine and adenosine remaining after the addition of di-Pt as a function of time (see Fig. 4) an estimation of the half-life of the reaction can be obtained. Di-Pt reacts with guanosine two-fold slower ($t_{1/2}$: 1 h) than cisplatin ($t_{1/2}$: 0.5 h) but considerably faster than with adenosine ($t_{1/2}$: 7 h).

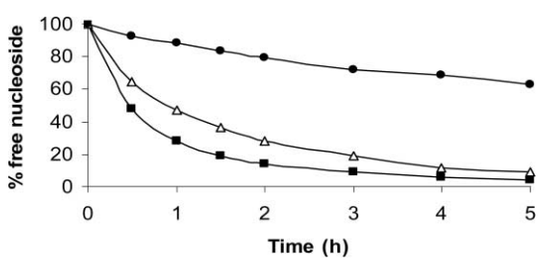

Fig. 4 The rate of reaction of cisplatin and di-Pt with the nucleosides: guanosine and adenosine, at 60 °C, plotted as a function of concentration of free base per hour. Cisplatin/gua (■), di-Pt/gua (△) and di-Pt/ade (●).

During the reaction the H5/H3 resonances of di-Pt (7.6 ppm) slowly decrease in size, with many new metal complex resonances appearing between 7.8 and 8.0 ppm, but again due to the complicated spectrum, no assignments can be made to particular metal complex adducts. Also consistent with covalent binding at the N7 and N1/N3 sites is the disappearance of the H1′ doublet (6.09 ppm) in conjunction with the appearance of new doublets between 6.1 and 6.3 ppm. Over six different doublets are observed, suggesting a mixture of N7 and N1/N3 binding.

Binding at N7 and at N1/N3 is also suggested by the ^{195}Pt NMR spectrum where two large different PtN_4 resonances are observed at −2486 and −2548 ppm (see Fig. S3, ESI †) as well as two small resonances around −2410 and −2440 ppm. Based on the ^{195}Pt NMR shifts observed in the reaction with guanosine (−2467 ppm), the resonance at −2486 ppm could be assigned to di-Pt bound at the N7 of adenosine, with the upfield resonance at −2548 ppm and the other two small resonances as the PtN_4 of the di-Pt bound at the N1/N3 sites.

Di-Pt/d(ATGCAT)$_2$ binding

After addition of di-Pt to the oligonucleotide d(ATGCAT)$_2$ at 37 °C the reaction was followed by ^{1}H NMR in order to investigate the ability of di-Pt to form 1,2-GG DNA interstrand cross-links. After reaction for 48 h and purification by HPLC (retention time 22 min) the major product of the reaction was found to be a 1,2-GG interstrand cross-link.

The assignment of the proton resonances from the oligonucleotide and metal complex were made from NOESY and DQFCOSY experiments.[15–17] Binding of the metal complex to the N7 of the G bases results in large chemical shift changes of selective oligonucleotide resonances (Table 2). Large downfield shifts are observed for the G_3H8 and C_4H6 proton resonances of 0.63 and 0.24 ppm, respectively (Fig. 5). The chemical shift change of ~0.6 ppm of the GH8 is consistent with the metal complex binding at the N7 of the guanine base, based on the results of the guanosine binding experiments and previous studies.[12–14] Large chemical shift changes are also seen for the T_2H6, T_6H6 and the A_5H2 resonances (0.14, 0.14 and 0.16 ppm, respectively). Downfield chemical shifts are also observed for the H1′ resonances of the T_2, G_3 and C_4 protons. Upon binding, the chemical shifts of the metal complex proton resonances only change slightly. Both the H5 and H3 resonances move downfield 0.02 ppm while the –CH_2– resonances shifts upfield 0.08 ppm.

Spectra of the di-Pt bound oligonucleotide dissolved in 90% H_2O/10% D_2O were recorded to confirm that the DNA

Table 2 Chemical shifts (ppm) of the non-exchangeable proton resonances of $d(ATGCAT)_2$ after formation of the 1,2-GG interstrand cross-link by di-Pt. Numbers in parentheses indicate the difference between the chemical shift of the metal complex bound and free oligonucleotide resonances. Negative shifts indicate an upfield shift of the resonance. Large chemical shift changes (>0.2 ppm) are indicated in bold

Base	H8/H6	AH2	H1′	H2′	H2″	H3′	H4′
A1	8.20 (0.00)	8.02 (−0.03)	6.32 (0.04)	2.72 (0.02)	2.74 (−0.05)	4.86 (−0.04)	n.d
T2	7.50 (0.14)	—	**6.20 (0.31)**	2.27 (0.15)	2.45 (0.03)	4.88 (0.00)	n.d
G3	**8.56 (0.63)**	—	**6.17 (0.22)**	2.73 (0.07)	2.65 (−0.03)	4.94 (−0.04)	4.41 (0.03)
C4	**7.64 (0.24)**	—	**6.05 (0.21)**	1.91 (0.02)	2.37 (0.04)	4.81 (0.00)	4.20 (0.02)
A5	8.35 (0.01)	8.01 (0.16)	6.31 (−0.03)	2.80 (0.01)	2.83 (0.00)	4.99 (−0.04)	4.41 (−0.03)
T6	7.50 (0.14)	—	6.15 (−0.01)	2.27 (0.05)	2.27 (−0.07)	4.56 (0.01)	n.d

n.d = not detected.

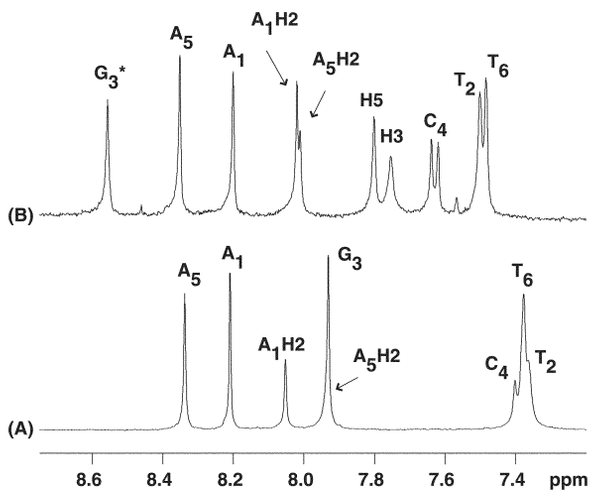

Fig. 5 ^{1}H NMR spectra of the aromatic region (A) the oligonucleotide $d(ATGCAT)_2$ and (B) the HPLC purified 1,2-GG interstrand cross-link, the major adduct formed by this oligonucleotide and di-Pt. G_3* denotes the platinum bound guanine residue.

remained in a double-helix conformation. Three imino resonances are seen, one much further upfield from where imino resonances from normal B-type DNA are observed. The resonances at 13.87 and 13.56 ppm are assigned to the A_1–T_6 and T_2–A_5 base-pairs and the resonance at 10.59 ppm to the G_3–C_4 base pair. The chemical shift of the G_3 imino is 2.14 ppm upfield from the G_3 imino of the free oligonucleotide. This shift is similar to that observed by Huang *et al.* for the formation of a DNA 1,2-GG interstrand cross-link by cisplatin.[18] This result shows that the oligonucleotide remains double stranded and suggests that a change in DNA conformation has occurred locally at the GpC binding site.

Analysis of the NOESY spectra of the di-Pt bound oligonucleotide shows several interesting features, particularly in the H8/H6–H1′ and H8/H6–H2′/H2″ regions. The expected sequential NOEs between the aromatic H8/H6 protons and the sugar H1′ protons are not observed, with only the NOE from each H8/H6 resonance to its own H1′ proton being observed. In the H8/H6 to H2′/H2″ region of the NOESY spectrum, only the sequential NOEs between the A_1 and T_2, G_3 and C_4 and A_5 and T_6 are observed (see Fig. S4, ESI †). Of most importance however, is the intensity of the G_3H8/H1′ NOE compared to the G_3H8/H2′ NOE. In normal B-type DNA the NOE to its own H2′ proton is larger than that to the H1′.[16] In the NOESY spectrum of the bound oligonucleotide the reverse is seen, indicating a possible local DNA conformation change. This reversal in the NOE intensities of the H8/H6–H1′/H2′ has been previously observed for guanine residues in 1,2-GG interstrand cross-links with cisplatin and the 1,1/*t*,*t* (n = 6) complex.[18,19] This was attributed to a rotation of the glycosidic bond for the guanine base from an *anti* to *syn* position. For the cisplatin adduct[18] this resulted in a change from B-type DNA to Z-type DNA and for the 1,1/*t*,*t* adduct[19] a change from B-type DNA to a dumb-bell shape. From the NOESY data it was not possible to determine the type of adduct formed by di-Pt, as no NOEs were observed from either the metal complex H5/H3 protons or the metal complex –CH_2– protons to any oligonucleotide proton.

The nature of the DNA conformational change was further examined through the analysis of DQFCOSY experiments. For B-type DNA the coupling (J) of the H1′ proton to the H2″ and the H2′ protons is 6 and 10 Hz, respectively, whereas for the 3′-endo sugar pucker of Z-type DNA the coupling constants are 8 and 2 Hz, respectively.[20] In the DQFCOSY spectrum of the di-Pt bound oligonucleotide stronger cross-peaks are observed from all oligonucleotide H1′ protons to the H2′ than to the H2″ proton that were resolved, except for the G_3 sugar. This is consistent with the general maintenance of the B-type DNA structure but with a partial (or an equilibrium) Z-type DNA local conformational change at G_3.

Transcription assay

The DNA binding of di-Pt and cisplatin were investigated using an *in vitro* transcription assay. Although the Tris buffer will compete with the DNA for binding to the platinum complexes,[21] previous studies have shown that incubation of cisplatin with either HEPES, sodium phosphate or TE buffer results in little observed difference in the reactivity and sequence specificity for cisplatin in subsequent transcription assays.[22–24]

Initial experiments established that overnight incubation of DNA with either 1 μM di-Pt (see Fig. 6) or 2 μM cisplatin was sufficient to completely inhibit the formation of a full-length transcript. Complete inhibition of the full-length transcript is observed at 10 μM, with the blockages so intense that transcripts are too short to be seen on the gel. Fig. 7 shows the relative amount of blockages at each site, from nucleotide 30 to nucleotide 118 (where 1 denotes the first nucleotide of the nascent transcript). The transcriptional sequence specificity of each of the compounds was determined from the transcriptional data. The sequence shown is that of the non-template strand and therefore reflects the same sequence as the RNA transcript produced. Importantly, the blockage sites produced by the two compounds were vastly different (Table 3). The two major blockage sites for cisplatin occur exactly one nucleotide prior to CC sequences, consistent with cisplatin intrastrand cross-links on GG dinucleotide sequences of the template DNA. In contrast, for the di-Pt complex the two blockage sites with the highest relative occupancy occur at the first A and T nucleotides in the G*AT*TA sequence. Additionally, while the next three most blocked sites occur one residue before a G or C, all three of these adducts may include binding at an adenine residue. While there are 7 GC (or CG) dinucleotides in the DNA segment analysed in the transcription assay, only one significant transcription blockage (occupancy greater than 3%) is observed at these potential 1,2-GG interstrand sites. Furthermore, no 1,2-GG intrastrand adducts are detected, and only one possible 1,3-GG intrastrand and one possible 1,3-GG interstrand adducts are observed.

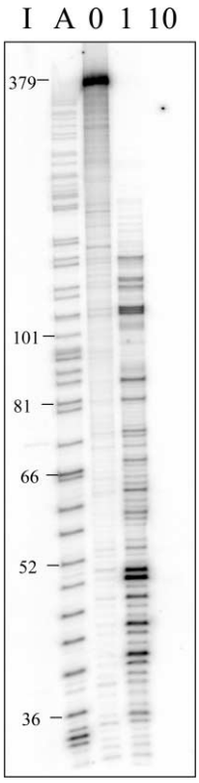

Fig. 6 A phosphorimage of transcriptional blockages induced by di-Pt following an *in vitro* transcription assay. DNA was reacted with 0, 1 or 10 μM di-Pt for 16 h at 37 °C prior to transcription initiation. Transcription was initiated by incubating drug-reacted DNA with *E. coli* RNA polymerase in transcription buffer. Initiated transcripts were elongated for 5 min at 37 °C. Lane A is a sequencing lane, which was obtained by adding the chain terminator 3′-*O*-methoxy-ATP during the elongation phase of transcription. Lane I is an initiation control which represents initiated transcripts that have not been elongated. Numbering represents the length of transcripts beginning from the first nucleotide.

Discussion

The DNA binding by the dinuclear platinum complex di-Pt has been examined using a transcription assay. Interestingly, the results showed that the two strongest transcription blockage sites occurred within an ATTA sequence. Additionally, while the next three strongest blockage sites occurred one nucleotide before a G–C base-pair, the G–C base-pair was located in an A/T rich region in two of the three cases. Based on the transcription assay results, it is concluded that di-Pt binds DNA with some preference for adenine bases over guanine bases.

While multi-nuclear platinum complexes have been shown to form a different array of DNA adducts to cisplatin, it has been generally established that, like cisplatin, they preferentially bind at guanine residues. This preference is thought to be due to the greater reactivity of guanine for the electrophilic platinum complexes. To confirm this kinetic preference, and establish that the di-Pt complex could not only form a mono-adduct but could also ring close to form bis-adducts at guanine residues, the binding of di-Pt with guanosine, adenosine and the oligonucleotide $d(ATGCAT)_2$ was studied.

The reaction of di-Pt with guanosine showed that the complex binds through the N7 position, as expected, with no evidence of binding at the N1 or N3 of guanosine. Furthermore, the observed stepwise substitution of chloride by guanosine is consistent with the mechanism of hydrolysis reported for the dinuclear platinum complex BBR3005,[7] where Cox *et al.* demonstrated that the monoaqua monochloro species is formed in the rate-limiting step before covalent binding to DNA, similarly to that observed for cisplatin.[7] This is broadly in agreement with the pyrazole ligand occupying a similar position to amines in the spectrochemical series,[25] with pyrazole being considered as essentially a σ donor.[26] Di-Pt was shown to bind to guanosine more rapidly than to adenosine, with $t_{1/2}$ = 1 and 7 h, respectively. Di-Pt was also shown to bind adenosine at the N7 site and at either the N1 or N3 sites. However, in duplex DNA where the N1 site is inaccessible due to Watson–Crick base-pairing, binding would only be possible at the N3 site. These results confirmed the kinetic preference of the di-Pt complex for guanine residues.

Di-Pt has been previously shown to predominantly form interstrand, rather than intrastrand, DNA cross-links.[10] Hence, in order to confirm that the di-Pt could ring close to form the bis-adduct at two guanine residues from an initial guanine mono-adduct, the binding of di-Pt to an oligonucleotide containing a potential 1,2-GG interstrand cross-link was examined. The major product in the reaction of di-Pt with $d(ATGCAT)_2$ was shown to be a 1,2-GG interstrand cross-link by NMR spectroscopy. Although a detailed study of the structure of the di-Pt bound oligonucleotide is beyond the scope of the present study, preliminary analysis indicated that the binding of the platinum complex significantly altered the structure of the oligonucleotide.

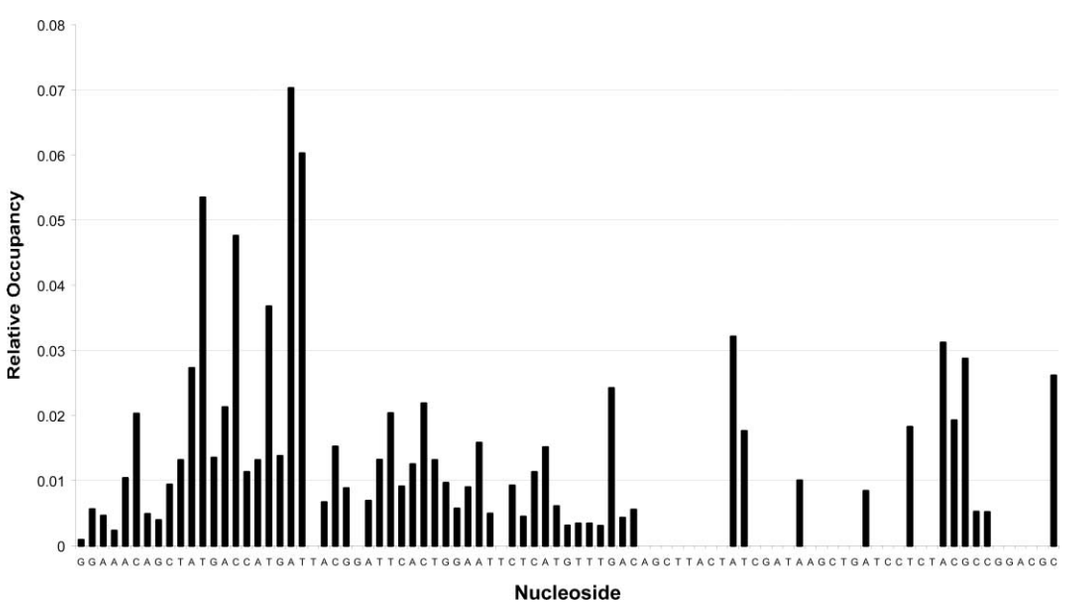

Fig. 7 Di-Pt induced blockages of transcription from the *lac* UV5 promoter after overnight incubation and the relative amount of each blockage (represented as mole fraction of total RNA of the lane) at each site of the non-template DNA sequence.

Table 3 The largest blockages induced by the di-Pt complex (1 μM) and cisplatin (2 μM) during *in vitro* transcription assays using the *lac* UV5 promoter. Underscored letters indicate the residue where the blockage begins

Metal complex	Relative occupancy	Sequence	Possible adduct type
Cisplatin	0.12	G*A*CCA	1,2 GG intra
Cisplatin	0.11	A*T*CCT	1,2 GG intra
Cisplatin	0.03	A*G*CTA	1,2 GA intra 1,3 GA inter
Cisplatin	0.03	C*G*CCG	1,2 GG intra 1,3 GG inter
Di-Pt	0.07	G*A*TTA	1,2 AA intra 1,3 AA inter
Di-Pt	0.06	A*T*TAC	1,2 AA inter 1,3 AG intra
Di-Pt	0.055	A*T*GAC	1,2 GA intra 1,3 GG inter
Di-Pt	0.048	A*C*CAT	1,2 GA inter 1,3 GA intra
Di-Pt	0.035	A*T*GAT	1,2 GA intra 1,3 GA inter
Di-Pt	0.03	T*A*TCG	1,2 AG intra 1,3 AG inter
Di-Pt	0.03	T*A*CGC	1,2 GG inter 1,3 GG intra

As di-Pt bound guanosine at a faster rate than adenosine and was shown to readily form a 1,2-GG interstrand cross-link with $d(ATGCAT)_2$, it is proposed that the preference shown for adenine residues in the transcription assay is due to the effect on the covalent binding reaction of the DNA duplex structure. Alternatively, it is possible that the transcription blockages are the result of non-covalently bound di-Pt. This, however, is unlikely as it has previously been shown that a much higher concentration of metal complex (50 μM) was required to cause blockage of DNA transcription by inert multi-nuclear platinum complexes.[8] In addition, the blockages induced by di-Pt are dissimilar to those caused by the inert di-Pt analogue $[(en)Pt(\mu\text{-}dpzm)_2Pt(en)]^{4+}$.[8] The largest blockages caused by $[(en)Pt(\mu\text{-}dpzm)_2Pt(en)]^{4+}$ occur mainly at G/C bases. Based on this information, it is unlikely that the transcription blockages observed with di-Pt are due to reversibly bound metal complex.

A more likely explanation for the observed preference for adenine residues in the transcription assay is the strong preference of multi-nuclear platinum drugs to associate in the DNA minor groove at A/T rich regions prior to covalently binding to DNA. In previous studies using inert analogues of di-Pt, we have demonstrated that the dpzm based platinum complexes preferentially associate at A/T rich regions rather than G/C rich regions by at least two orders of magnitude.[5,8] Qu *et al.* have recently proposed that the trinuclear platinum complex BBR3464 pre-associates in the minor groove with the terminal platinum centres then straddles the sugar phosphate backbone to bind at the N7 atom of two guanine residues.[9] Consequently, if the multi-nuclear platinum complex pre-associates in an A/T rich region, the terminal platinum centres will more likely encounter an adenine residue when it crosses over into the major groove. The higher local concentration of the reactive platinum centre at adenine residues may counter-act the kinetic preference for guanine residues, thereby resulting in a higher percentage of adenine adducts.

It is probable that di-Pt would show a greater preference for pre-association at A/T rich regions than the aliphatic linked BBR complexes, due to increased van der Waals interactions from the dpzm rings, and hence exhibit a greater preference for forming covalent adducts at adenine residues. While di-Pt displays good cytotoxic properties, the corresponding aliphatic di-nuclear complex (BBR3005) is more active in both platinum-sensitive and resistant cell lines.[10] This could be due to the higher percentage of adenine adducts formed by di-Pt, however, it is also possible that the difference in the cytotoxicity is due to the types of long-range adducts formed and their corresponding effect on the DNA structure. In particular, given its relatively rigid structure, di-Pt essentially only forms interstrand cross-links,[10] which may not cause cytotoxic lesions to the same extent as intrastrand cross-links. However, given that linking ligands containing aromatic groups and a greater degree of flexibility can be synthesised, it is possible that complexes capable of forming long-range intrastrand cross-links with a preference for adenine residues can be produced.

The results of this study further demonstrate the considerable potential of multi-nuclear platinum complexes, as it is possible that they can be designed to target particular DNA sequences in the major groove, and perhaps, given the reactivity of di-Pt for the N1/N3 of adenosine, in the DNA minor groove as well.

Experimental

Materials

The oligonucleotide was purchased from Geneworks, South Australia and prepared as previously described.[6] Transplatin, cisplatin, pyrazole and tetrabutylammonium hydroxide were purchased from the Aldrich Chemical Company. D_2O was purchased from Cambridge Isotope Laboratories. 5′-GMP, guanosine and adenosine, were purchased from Sigma. Nucleotides, *E. coli* RNA polymerase (nuclease free) and [α-^{32}P]UTP (sp. act. 3000 Ci mmol^{-1}) were purchased from Amersham Pharmacia Biotech (Amersham, UK). The dpzm ligand[27] and the metal complex[10] were made as previously described. All solvents were used as provided and aqueous solutions were made using Milli-Q water, coming from a Millipore four-stage water purification unit.

NMR

1D and 2D NMR spectra were obtained on a Varian Unity*plus-400* spectrometer operating at 400 MHz for ^{1}H and 85 MHz for ^{195}Pt. 1D spectra were recorded over a spectral width of 5000 Hz using 256 transients. T_1 relaxation experiments were acquired with a 180°–d_2–90° pulse sequence. 2D spectra were obtained over a spectral width of 4200 Hz with mixing times of 100, 250 and 300 ms using 2048 points in the t_2 dimension with 256 t_1 increments and with a total recycle time of 1.7 s. 1D and 2D NMR experiments carried out in 90% H_2O/10% D_2O were run using the WATERGATE (Water suppression by GrAdient-Tailored Excitation) solvent suppression technique of Piotto *et al.*[28] All ^{1}H NMR experiments were referenced to DSS (0 ppm) at 25 °C. ^{195}Pt NMR experiments were externally referenced to K_2PtCl_4 at −1631 ppm and collected using a spectral

width between 75000 and 100000 Hz, with between 1000 and 10000 transients and a recycle time of 0–0.4 s.

HPLC

A Bio-rad 2800 solvent delivery system was used, coupled to a Hewlett Packard HP 1090L liquid chromatograph with a diode array detector, with flow rate of 1 mL min^{-1} for the Zorbax eclipse XBD-C8 analytical column and a flow rate of 4.176 mL min^{-1} for the Zorbax eclipse XBD-C8 semi-prep (9.4 mm by 25 cm) column, set at 40 °C. The gradient used was based on those developed by Hambley and Munk[29] using a dual solvent delivery of solvent A (50% H_2O/50% CH_3CN) and solvent B (TEAA 20 mM). Gradient: Time 0 min: A 12, B 88%; 9 min: A 12, B 88%; 18 min A 17, B 83%; 27 min: A 17, B 83%; 30 min A 100, B 0%; 40 min: A 100, B 0%, 40.1 min A 12, B 88%; 50 min: A 12, B 88%.

Nucleoside experiments

The nucleosides were dissolved in 4550 μL of D_2O and heated at 60 °C (± 2 °C) in a water bath. The metal complex (2 equivalents) was added to give a final metal complex concentration of 18 mM and allowed to react over 24 h. Aliquots (650 μL) were taken at various time intervals and ^1H NMR spectra obtained, ensuring the samples were kept at 60 °C at all times. After analysis the aliquots were returned to the sample. The solutions were kept unbuffered to prevent reactions between the metal complexes and buffer. After 24 h the solution was freeze dried then concentrated in 650 μL of D_2O for ^{195}Pt NMR analysis.

In vitro transcription assays

A 512 bp fragment of DNA (25 μM bp) containing the *lac* UV5 promoter, prepared as previously described,[8] was incubated for various time periods at 37 °C in 0.5 × TE buffer (1 × TE; 10 mM Tris, 1 mM EDTA, pH 7.0) in the presence of either di-Pt or cisplatin. The samples were then subjected to a transcription assay.[23] The drug-reacted DNA was added to a transcription mix containing *E. coli* RNA polymerase and non-specifically bound polymerase was removed by addition of heparin. Synchronised initiation transcripts were formed using the dinucleotide GpA, [α-^{32}P]UTP, GTP and ATP, with elongation being facilitated by the addition of 2 mM concentrations of all four ribonucleotides for 5 min before the addition of an equal volume of loading termination buffer (10 M urea, 40 mM EDTA, 10% sucrose, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 × TBE). Samples were denatured at 90 °C for 5 min and then electrophoresed through 12% denaturing polyacrylamide sequencing gels. Analysis was performed using ImageQuaNT software after digital capture of radiographic images using a Molecular Dynamics 400B PhosphorImager.

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