Platinum Binding Preferences Dominate the Binding of Novel Polyamide Amidine Anthraquinone Platinum(II) Complexes to DNA

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Abstract:

Complexes incorporating a threading anthraquinone intercalator with pyrrole lexitropsin and platinum(II) moieties attached were developed with the goal of generating novel DNA binding modes, including the targeting of AT-rich regions in order to have high cytotoxicities. The binding of the complexes to DNA has been investigated and profiles surprisingly similar to that for cisplatin were observed; the profiles were different to those for a complex lacking the pyrrole lexitropsin component. The lack of selective binding to AT-rich regions suggests the platinum binding was dominating the sequence selectivity, and is consistent with the pyrrole lexitropsin slowing intercalation. The DNA unwinding profiles following platinum binding were evaluated by gel electrophoresis and suggested that intercalation and platinum binding were both occurring.

Keywords

Pyrrole lexitropsins – Anthraquinone – Platinum amidine complexes – Monofunctional adducts – DNA binding

Introduction

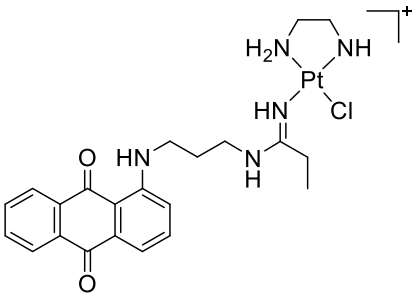
Ligand promoted selective accumulation in cancer cells of highly cytotoxic drugs has the potential to reduce their numerous and severe side-effects and enable more effective treatments of those cancers.[1] This is particularly important for platinum drugs because they are among the most widely used anticancer agents, but cause both short- and long-term harm to cancer patients.[2-4] For ligand promoted accumulation to be effective, it has been suggested that the drug being delivered should have a cytotoxicity in the low nanomolar range because of the rate-limited capacity of the uptake pathways.[5] Some highly lipophilic platinum(IV) complexes derived from close analogues of existing drugs do have IC_{50} values in this range[6] and therefore more selective means of promoting cellular accumulation may be effective.[1] However, platinum complexes with still lower IC_{50} values would be better candidates for making selective accumulation effective.

We have recently reported on an attempt to generate much more toxic platinum drugs by incorporating both intercalating and minor groove binding components into a single complex.[7] The design rationale was that incorporation of pyrrole lexitropsins would promote binding to AT rich regions of DNA, a feature that is associated with extraordinary cytotoxicity for compounds such as bizelesin and adozelesin.[8, 9] Bierbach and colleagues have reported that platinum complexes which bind to adenine in the minor groove show remarkable cytotoxicity[10, 11] and Aldrich-Wright and colleagues have used lexitropsins to modify the sequence selectivity of platinum complexes.[12, 13]

Our complexes combined the amidine linked intercalator motif developed by Bierbach with the polyamide motif using a 1,5-anthraquinone as a threading intercalator in the expectation that the polyamide would bind in the minor groove directing the platinum to the major groove.[7] The cellular accumulation and cytotoxicity of the complexes [PtCl(am1C3)(en)]Cl **1** and [PtCl(*x*py-am1,5C3)(en)]Cl, *x* = 1-3, **2a-c** (Chart 1) were investigated and they were found to have potency similar to that of cisplatin, a disappointing and surprising result. Therefore, we were interested in investigating the DNA binding preferences of these complexes to help understand the causes of the lower than hoped for cytotoxicity.

The preferred site of DNA binding is likely to be influenced by one or more of the three different DNA binding moieties and the combination of binding mechanisms was expected to lead to novel DNA damage.[10, 14, 15] The possible binding mechanisms of the platinum

amidine unit differ from that of cisplatin in that the amidine complex is not a cross-linking agent.[10, 16] It was anticipated that the selectivity of the coordinative DNA interaction would be dominated by the sequence selectivity of the anthraquinone and/or the polyamide lexitropsin since these rely on non-covalent interactions which are established more rapidly. To characterize the location and nature of platinum binding to dsDNA, DNA binding studies were undertaken. Footprinting studies using a polymerase stop assay were used to determine the sequence at which the adducts are formed with good nucleotide resolution,[17, 18] and an unwinding assay was used to provide insights into the impact of the binding of the complex to the DNA.



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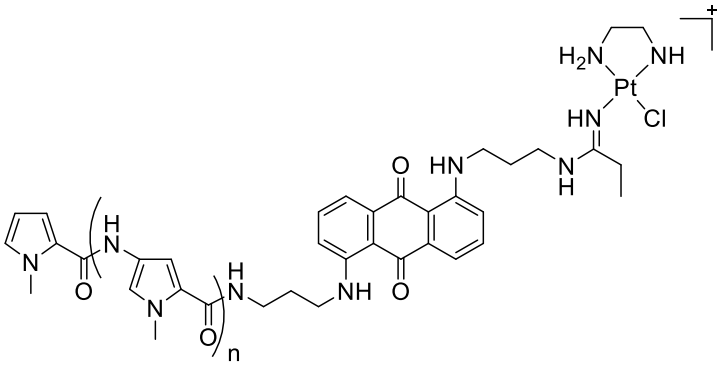

n = 0 **2a**
n = 1 **2b**
n = 2 **2c**

Experimental

Materials

cis-Diamminedichloridoplatinum(II), ethidium bromide (EtBr), bromophenol blue, DMF, sodium acetate, and hydrochloric acid (37 wt.% in H_2O) were purchased from Sigma-Aldrich, Pty., Ltd, Australia. Absolute ethanol, and EDTA were purchased from Ajax Chemicals, Australia. Agarose was purchased from AppliChem. Deoxynucleotide triphosphates (dNTPs) were purchased from the Ramaciotti Centre, UNSW. Ultrapure agarose low electroendosmosis (EEO) was purchased from Invitrogen. All commercial reagent grade chemicals were used without further purification. The preparation of the complexes has been reported previously.[7]

TE buffer: 2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl) and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); TAE buffer: Tris base, glacial acetic acid, and EDTA were purchased from Ajax Finechem.

Membrane for dialysis Slide-A-Lyzer MINI Dialysis Unit 10K Molecular Weight Cut Off was purchased from Thermo Fisher Scientific.

Enzymes, *Pvu*II and *Hinf*I were obtained from New England Biolabs. AmpliTaq® DNA polymerase was purchased from Life Technologies. All enzymes and associated buffers were used as recommended by the manufacturer.

Oligonucleotide primers fluorescently tagged with 6-carboxyfluorescein (6-FAM) were supplied by Invitrogen. The sequences used for linear amplification of damaged DNA were (6-FAM)-Seq2 ((6-FAM)-5'-ATGTGCTGCAAGGCGA-3') or (6-FAM)-Mito15 ((6-FAM)-5'-ACTCAACATACTAGTCAC-3').

Instrumentation

A Nanodrop 1000 spectrophotometer (Thermo Scientific version 3.6.0) was used to determine DNA concentration in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0). Gels stained with ethidium bromide were imaged using a BioRad UV transilluminator. Images of gels were viewed using Quantity One version 4.6.6.

Platinum concentrations were determined by an Agilent 200 Series Graphite Furnace Atomic Absorption Spectrometer, with a GTA 120 Graphite Tube Atomiser and an UltrAA Boosted Lamp Supply.

DNA Damage Studies

Two different clones, $T_7G_{10}CpG$ and the HMHVR clone were made by modifying a pUC19 plasmid, using a previously described process.[19, 20] These clones were used to determine the sequences at which the platinum conjugates bind.

The plasmid was digested with *Pvu*II to provide consistent length DNA fragments during linear amplification prior to use in platinum complex damage experiments. This also generated a consistent concentration (120 ng/µL) of digested plasmid. Gel electrophoresis was used to determine whether the plasmid was fully digested by *Pvu*II.

Ethanol Precipitation of DNA

Ethanol precipitation of DNA (EtOH/H_2O 70%) was used for concentrating and purifying DNA samples. Sodium acetate (3 M, 1:9 v/v wrt sample volume) was added to the DNA sample. The sample was briefly mixed, then absolute ethanol was added to give a final concentration of 70% v/v. Samples were briefly mixed again and stored at -20 °C overnight. Samples were centrifuged at 16000×*g* at 4 °C for 25 min to recover the precipitated DNA. The DNA pellet was washed with an ethanol/water mixture (7:3 v/v, 2 × 150 µl). The pellet was dried *in vacuo* using a Speedivac vacuum desiccator at rt for approximately 10 min. or until it was dry. The dried DNA pellet was dissolved in 10/0.1 TE buffer (10 mM Tris-HCl, 0.1 mM EDTA at pH 8.0).

Linear Amplification

Thermal cycling was used with the FAM-labelled primer Seq2 (5'- ATGTGCTGCAAGGCGA-3') or Mito15 (5'-ACTCAACATACTAGTCAC-3') to amplify a specific region of interest that started with the complementary strand (3'-TACACGACGTTCCGCT-5') or (3'-TGAGTTGTATGATCAGTG-5') respectively within the plasmid. In the PCR sample, 48 ng of plasmid DNA was used per 10 pmol of (6-FAM)-Seq2 primer, and to this 20× PCR buffer (1 µL, with a final concentration of 16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8), and 6.7 mM $MgCl_2$), 6 nmol of deoxynucleotide triphosphates (dNTPs), and 1U *Taq* DNA polymerase were added. The volume was then made up to 20 µL with MilliQ water. Thermal cycling involved incubation at 95 °C for 45 s to denature the dsDNA into ssDNA, then annealing the (6-FAM)-Seq2 or (6-FAM)-Mito15 primer to the ssDNA at 55 °C for 60 s. The DNA was then extended by *Taq* DNA polymerase

at 72 °C for 75 s, and a new DNA strand was synthesised complementary to the ssDNA strand in a 5’ to 3’ direction. The cycle was repeated 20 times and the last extension step was held at 72 °C for 5 min to ensure all the ssDNA was fully extended. The sample was held at 4 °C for at least 5 min.

Dideoxyribonucleotide Sequencing

Thermal cycling was used with the 6-FAM-labelled primer Seq2 or Mito15 to amplify DNA fragments using a mix of dNTPs and dideoxynucleotides. This ensured that the syntheses of the complementary strands terminated after incorporating the dideoxynucleotide. In the PCR sample (20 µL reaction), 48 ng of plasmid DNA was used per 10 pmol of (6-FAM)-Seq2/Mito15 primer, and 20× PCR buffer was added, followed by 1-2 mM of either ddATP, ddTTP, ddCTP, or ddGTP, 50 µM dNTP, 1U of *Taq* DNA polymerase. The volume was then made up to 20 µL with MilliQ water. The thermal cycling conditions involved incubation at 95 °C for 30 s to denature the dsDNA into ssDNA, annealing the (6-FAM)-Seq2/Mito15 primer to the ssDNA at 55 °C for 60 s, and extension of DNA by *Taq* DNA polymerase at 72 °C for 75 s. This generates a new DNA strand complementary to the ssDNA strand in a 5’ to 3’ direction with the DNA fragment bearing the 3’-H. The cycle was repeated 15 times and the last extension step was held at 72 °C for 5 min to ensure all of the ssDNA was fully extended. The sample was held at 4 °C for at least 5 min. The dideoxynucleotide sequencing products were washed using the ethanol precipitation protocol.

Gel Electrophoresis

Gel electrophoresis was used to confirm whether the plasmids were fully digested by *Pvu*II. DNA samples from the digest were loaded with 2× loading dye (0.1% w/v bromophenol blue/glycerol) and were electrophoresed on 2.0% w/v agarose gels with 1× TAE running buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 100 V for 1 h. They were then stained with ethidium bromide (1 µg/mL) and washed with MilliQ water. The gel was visualised with a UV transilluminator (GeneGenius) by Syngene.

Plasmid Damage Studies

*Pvu*II digested plasmid (240 ng) was added to 10× buffer H (2 mM HEPES, 10 mM NaCl, 10 mM EDTA, pH 7.8), and varying concentrations of drug ranging from final concentrations of 0.1 µM to 3 µM. The volumes were made up to 20 µL with MilliQ water. The samples were incubated at 37 °C for 6.75 h. in the dark. The drug damaged DNA was precipitated as per the

ethanol precipitation of DNA protocol. The DNA pellet was redissolved in 10/0.1 TE buffer (10 µL) and stored at -20 °C for use in linear amplification procedures.

Linear Amplification of Damaged DNA

Each linear amplification reaction was carried out using the thermal cycling protocol. The 20 µL thermal cycled product was transferred to a 1.5 mL Eppendorf tube and NaOAc (3 M, 2 µL) was added to the sample, followed by cold absolute ethanol (45 µL). DNA precipitation was carried out at 4 °C overnight and the DNA was washed using the ethanol washing procedure. The dried linear amplified DNA was dissolved in 10/0.1 TE buffer (10 µL), mixed thoroughly, and submitted for fragment analysis.

Fragment Analysis

An aliquot of the platinum damaged *Pvu*II cleaved 6-FAM labelled DNA (2 µL) was sent for fragment analysis to the Ramaciotti Centre for Gene Function Analysis at UNSW. The Centre added a mix of LIZ500 marker (20 µL) and Hi-Di formamide to the platinum damaged aliquot, and 15 µL of the mixture was loaded onto a 96 well plate. Samples were denatured at 95 °C for 5 min, followed by injection into an ABI3730 capillary sequencer at 1.6 kV for 15 s. The sequencing run was performed at 63 °C for 35 min.

Data Analysis

Fragment analysis data was processed with GeneMapper software from Applied Biosystems. The fluorescence trace data was evaluated using an algorithm developed by Murray and co-workers, to generate plots on electropherograms.[18] The plots were used to quantify platinum damage sites.

Plasmid DNA Unwinding Assay

Plasmid DNA unwinding assays were used to establish the mode of platinum-DNA binding. pTOPO-EIRE DNA (6097 bp, 50% GC content, 2 µg) was treated with the platinum complexes in 1× TE buffer for 24 h at 37 °C at concentrations ranging from 0 to 10 µM in 56 µL reaction volumes. Afterwards, the samples were dialysed with 1× TE buffer (3 L) at 4 °C. Portions from each sample (10 µL, approximately 0.3 µg of DNA) were combined with loading dye (blue, purple and orange, 6×, 2 µL) and electrophoretically chromatographed on 1.0% agarose gel in the absence of ethidium bromide in 1× TAE buffer.

The gel was immersed in 0.5 µg/mL ethidium bromide for approximately 20 min, followed by destaining with MilliQ water for 20 mins and then photographed. Final bound platinum concentrations were measured by GF-AAS.

Ethanol Precipitation of DNA

The plasmid DNA was incubated with the platinum agent for 24 h and the samples were transferred to a Slide-A-Lyzer MINI dialysis membrane and dialysed in 1× TE buffer for 24 h. Absolute ethanol was added (120 µL) and the DNA was kept at 4 °C overnight to allow precipitation. The samples were centrifuged at 16000×*g* at 4 °C for 25 min to recover the precipitated DNA. The supernatant was removed, followed by washing of the DNA pellet with the ethanol/water mixture (7:3 v/v, 2 × 150 µL). The pellet was dried *in vacuo* using a Speedivac vacuum desiccator at rt for approximately 10 min or until it was dry. The dried DNA pellet was dissolved in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0).

Gel Electrophoretic Mobility Shift Assay

Gel electrophoresis was used to observe the migration of supercoiled DNA towards the fully relaxed form of DNA upon increasing platinum concentration. The redissolved DNA samples were run on 1% w/v agarose gels in 1x TAE running buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) with 2× loading dye (0.1% w/v bromophenol blue/glycerol). The gel was electrophoresed with 1× TAE running buffer at 120 V. The gel was stained with ethidium bromide (1 µg/mL) in 1× TAE buffer. The gel was visualised with a UV transilluminator.

Graphite Furnace

The remainder of the redissolved DNA samples were used for the determination of DNA-bound platinum. Sample preparation for GF-AAS involved the digestion of the DNA samples with HNO_3 (69%, AAS grade, 200 µL) and sonciation in an ultrasonic bath for 2 h. The digested samples were diluted to 500 µL using MilliQ water. The GF-AAS instrument was calibrated using known platinum concentrations of K_2PtCl_4 and the platinum content was determined.

Results and Discussion

Sequence selectivity

The sequence selectivity of platinum binding to DNA was determined using plasmids with a known number and sequence of nucleobases (Figures S1 and S2). T_7G_{10}CpG and HMHVR clones were digested with *Pvu*II producing 396 bp and 568 bp fragments respectively (Figure S3). The bands corresponding to the undigested plasmids disappeared confirming completion of the digest. The DNA fragments were incubated with a range of concentrations of the platinum agents for sequence selectivity experiments. The primers used in this study (6-FAM Seq2 for T_7G_{10}CpG; 6-FAM Mito15 for HMHVR) corresponded with sequences unique to the smaller fragment of DNA.

Taq DNA polymerase was used to synthesise DNA until the enzyme encountered a platinum adduct. To determine whether the *Taq* DNA polymerase stopped extending once it reached a platinum damaged site rather than a bulky polyamide-anthraquinone moiety, the platinum free 3py-1,5C3 group was used. The fragment analysis showed no fragment peaks suggesting that this compound did not stop *Taq* DNA polymerase from extending DNA. Therefore, the fragments produced were generated by platinum binding to DNA. This linear amplification assay determines with good sensitivity the DNA sequence at which the platinum complexes bind. However, the optimal concentration of each platinum complex had to be determined as excessive damage due to a high platinum concentration will produce shorter fragments leading to meaningful information being lost, and too little damage due to a low platinum concentration will lead to a large amount of the full fragment being synthesised and adduct peaks being too close to the background to be detected reliably.

Platinum binding to the T_7G_{10}CpG/P*vu*II region

Platinum adduct formation was first investigated using the *Pvu*II cleaved pUC19 plasmid with a T_7G_{10}CpG insert. Cisplatin was used as a control with this plasmid as it produces a reproducible pattern of damage at the GpG sequences of the plasmid. Since the novel polyamide-anthraquinone-platinum complexes produce monofunctional platinum adducts with DNA, transplatin, which generates monofunctional adducts, was also used as a control. All of the complexes were used at concentrations ranging from 0.1 to 3.0 µM. The platinated samples were then used as templates for linear amplification and analysed to reveal the sequences to

which the complexes bind preferentially. The electropherogram of the full extension product is shown in Figure S4.

A large number of fragment peaks was observed between bases 0 to 160 in the electropherogram of the *Pvu*II digested T_7G_{10}CpG plasmid (Figure S4). This is due to the increased likelihood of the first platinum damage encountered by *Taq* DNA polymerase occurring toward the beginning of the plasmid. A comparison of the electropherogram profile of the cisplatin treated plasmid with those treated with the polyamide-anthraquinone-platinum complexes revealed that they have similar profiles as indicated by the dashed purple lines. This result was surprising given that these novel platinum complexes were designed to target different sequences of DNA. There are some DNA sequences that had stretches of A/Ts followed by GG so the platinum complexes could share the same adduct peak as cisplatin, but the electropherograms show that the platinating agents were binding at areas of the plasmid with high GC content and where there were fewer runs of A/Ts (Figure S4). The $[1C3\text{-}Pt(II)]^+$ complex was found to exhibit a significantly different damage profile to cisplatin and the other polyamide-anthraquinone-platinum complexes as indicated in Figure S4 (red box). The electropherogram generated by treatment with $[1C3\text{-}Pt(II)]^+$ showed extra peaks which are illustrated with an asterisk (Figure S5a). The corresponding DNA sequence (from dideoxy sequencing) was aligned with the zoomed overlay (Figure S5b) and the sequence was identified as 3'-(A/T)GTA-5'. The electropherogram did not show peaks at the sequence 3'-CGTA-5', indicating that $[1C3\text{-}Pt(II)]^+$ has high selectivity for the presence of an A or T in the 3' position.

Platinum binding the Mito15/P*vu*II region.

Having identified the binding of $[1C3\text{-}Pt(II)]^+$ to the 3'-(A/T)GTA-5' sequence with the T_7G_{10}CpG/*Pvu*II plasmid, platinum adduct formation was investigated using a *Pvu*II cleaved pUC19 plasmid with a HeLa mitochondrial hypervariable region (HMHVR) insert. This plasmid with the insert was chosen as it also had multiple 3'-(A/T)GTA-5' sequences and a 3'-CGTA-5' sequence (Figure S6). The polyamide-anthraquinone-platinum complexes were found to cause similar damage to each other and to cisplatin (Figure S6), reminiscent of the binding to the T_7G_{10}CpG plasmid. The $[1C3\text{-}Pt(II)]^+$ complex was found to damage the 3'-(A/T)GTA-5' sites and again no binding occurred at the 3'-CGTA-5' sequence (Figure S7). This result is significant as it shows that the fragment peaks occur at the same sequence in different positions on different plasmids. The $[1C3\text{-}Pt(II)]^+$ and $[Py\text{-}1,5C3\text{-}Pt(II)]^+$ complexes

were expected to give different damage profiles due to differences in the interactions of the intercalator to DNA. The 1C3 group has an aryl hydrogen at the 5 position of the anthraquinone whereas the 1,5C3 group has a NH hydrogen bond donor at the same position. It was also expected that the 1,5C3 group would prefer to intercalate at GG or GC sites thus directing the platinum to damage to GG regions. The damage experiments showed that the polyamides of the $[Py\text{-}1,5C3\text{-}Pt(II)]^+$ complexes did not direct the platinum to attach to A/T rich sequences and raises the question as to whether the AAQ is intercalating in DNA after the platinum adduct has formed.

The *Taq* DNA polymerase assay is capable of distinguishing between monofunctional cisplatin adducts and crosslinks.[21] Transplatin was used as a plasmid damage control as the complex produces monofunctional platination of DNA similar to that expected for the polyamide-anthraquinone-platinum(II) complexes **1** and **2a-c**. Transplatin was found to have a displaced damage position towards the 5'-end with an extra adenine present after the platinum adduct damage (i.e. showing a fragment corresponding to thymine (asterisk) 3'-GT*-5' on the complementary strand). The *Taq* DNA polymerase approaches from the left in the above sequence and preferentially adds an adenine to the 3' end of the product as it extends nucleotides from 5' to 3' of DNA (Figure 3).[22]

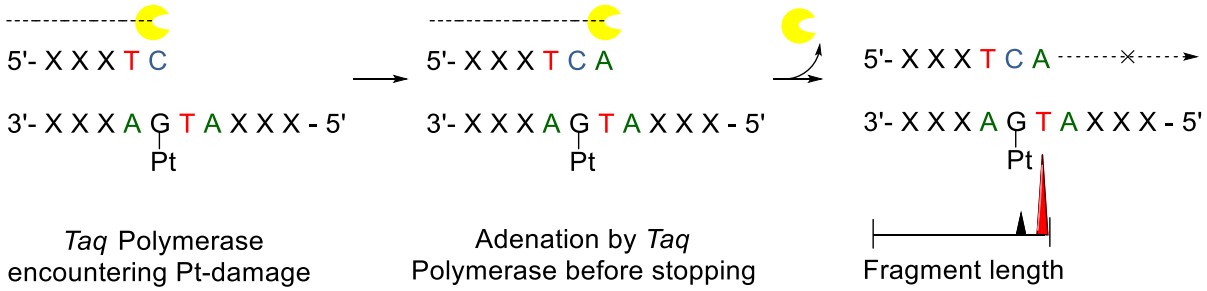

Figure 3. *Taq* DNA polymerase synthesising an extra adenine after encountering site of platination for monofunctional platinum(II) complexes.

Murray and co-workers have reported that bulky phenanthridinium conjugated to platinum(II) complexes can also inhibit *Taq* DNA polymerase activity. These complexes were shown to stop *Taq* DNA polymerase 2 base pairs before the usual stop sites for cisplatin and it was reasoned that the *Taq* DNA polymerase encountered the phenanthridinium group before the platinum adduct.[23] $[1C3\text{-}Pt(II)]^+$ did not exhibit a shift in damage to the plasmid which may be due to either the bulky anthraquinone moiety not intercalating in the DNA or the platinum lesion being encountered by *Taq* DNA polymerase before the anthraquinone moiety. To

investigate further, DNA unwinding experiments were used to determine whether the AAQ participates in intercalation.

DNA Unwinding Studies

Platinum complexes that form a covalent interaction with DNA have been shown to induce positive supercoiling, unwinding negatively supercoiled DNA as a result. The gel electrophoretic mobility shift assay was used in this investigation to determine the amount of the platinum intercalator complexes needed to completely unwind supercoiled DNA. After incubation of the compounds bearing the anthraquinone moiety with the supercoiled DNA, the mixture was dialysed with a semipermeable membrane. Platinum free compounds bearing the AAQ group were used as controls.

The unwinding angle is determined from the point at which there is co-migration of the relaxed and supercoiled bands of DNA (Figure 4).[24] The amount of the platinum at the coalescence point was determined from GF-AAS and the unwinding angle for the complex was calculated.[24] The unwinding angle caused by the anthraquinone-platinum(II) series was found to be 11° which is less than the 13° reported for cisplatin,[25] but larger than the unwinding angle caused by previously reported platinum complexes that bind monofunctional complexes.[24] The extra DNA unwinding is consistent with the intercalation of the anthraquinone moiety in the DNA helix. Hence, it is likely that the anthraquinone-platinum(II) complexes have a cooperative intercalative-platinating binding mode. A similar finding was reported by Keck and Lippard.[24] It was reasoned that the unwinding contribution of the intercalating group is smaller than expected due to the unfavourable constraints resulting from the coordination of the platinum-intercalator hybrid complex to DNA.[24] We have previously reported that the fluorescence of **1** and **2a-c** is lost in the presence of DNA,[7] providing further evidence of an intercalative binding mode.

The similarity of the unwinding angles for **1** and **2a-c** suggest that they have similar modes of interaction with DNA which presumably involves intercalation. It also shows that the presence and length of the polyamide group exerts little or no influence on the extent of unwinding.

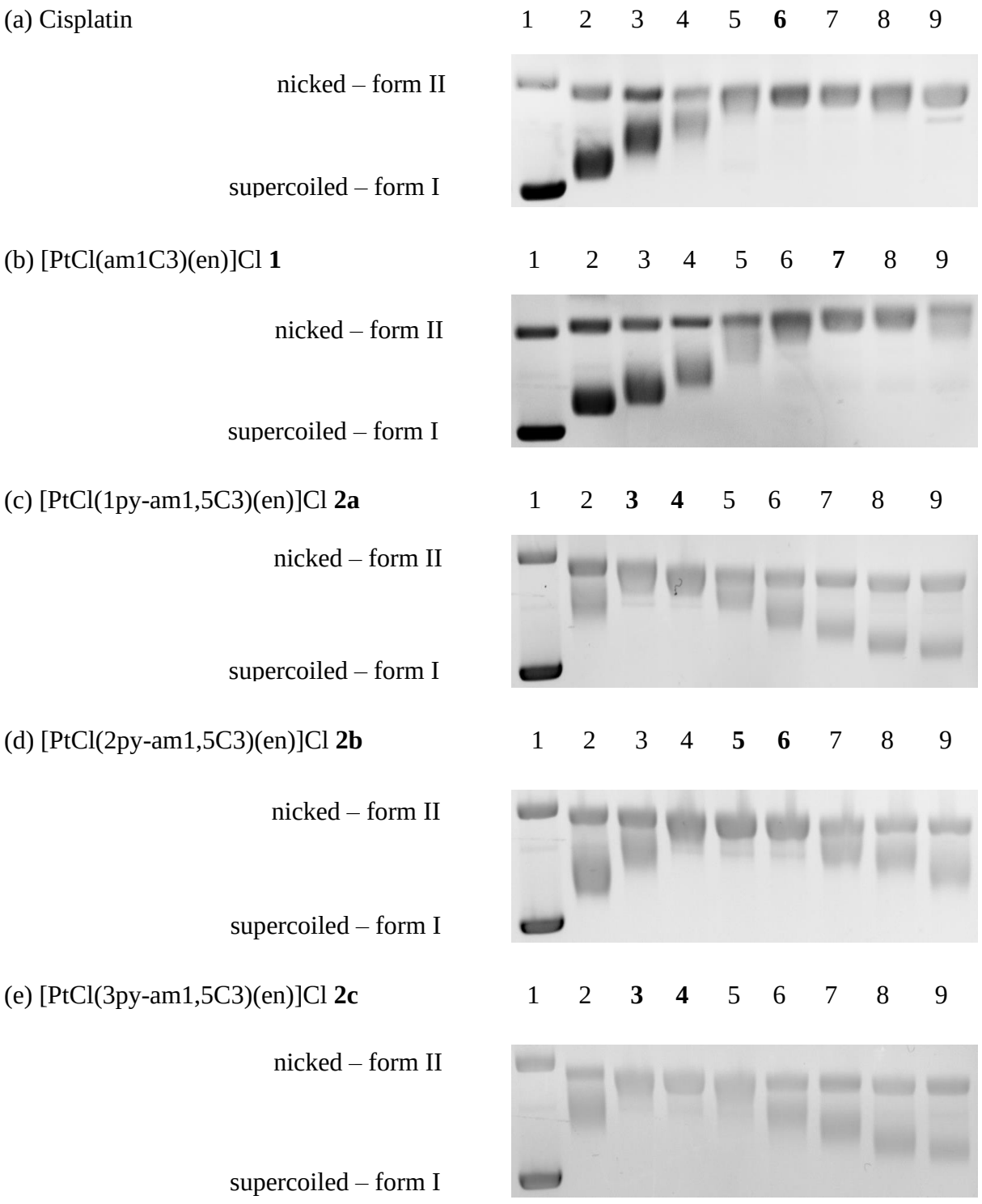

Figure 4. Titration of pTOPO-EIRE DNA: A 1.0% agarose gel showing the electrophoretic mobility of pTOPO-EIRE DNA which has been treated with (a) cisplatin, r_b levels for lanes 1-9, (1) 0.0, (2) 0.044, (3) 0.060, (4) 0.074, (5) 0.090, (6) 0.099, (7) 0.110, (8) 0.119, (9) 0.130; (b) [PtCl(am1C3)(en)]Cl **1**, r_b levels for lanes 1-9, (1) 0.0, (2) 0.051, (3) 0.064, (4) 0.088, (5) 0.095, (6) 0.107, (7) 0.116, (8) 0.130, (9) 0.162; (c) [PtCl(1py-am1,5C3)(en)]Cl **2a**, r_b levels for lanes 1-9, (1) 0.0, (2) 0.083, (3) 0.107, (4) 0.128, (5) 0.167, (6) 0.183, (7) 0.206, (8) 0.232, (9) 0.253; (d) [PtCl(2py-am1,5C3)(en)]Cl **2b**, r_b levels for lanes 1-9, (1) 0.0, (2) 0.058. (3) 0.078, (4) 0.092, (5) 0.108, (6) 0.122, (7) 0.130, (8) 0.159, (9) 0.184; (e) [PtCl(3py-am1,5C3)(en)]Cl **2c**, r_b levels for lanes 1-9, (1) 0.0, (2) 0.084, (3) 0.106, (4) 0.130, (5) 0.144, (6) 0.158, (7) 0.174, (8) 0.194, (9) 0.207.

Table 1. Unwinding of plasmid DNA by Platinum Complexes

Compounds	r_b(c)	Unwinding angle, deg
Cisplatin	0.099	13[26-28]
$[Pt(NH_3)_3Cl]Cl$		6 ± 1[24]
[Pt(dien)Cl]Cl		6 ± 1[24]
[PtCl(am1C3)(en)]Cl **1**	0.116	11 ± 1
[PtCl(1py-am1,5C3)(en)]Cl **2a**	0.117	11 ± 1
[PtCl(2py-am1,5C3)(en)]Cl **2b**	0.115	11 ± 1
[PtCl(3py-am1,5C3)(en)]Cl **2c**	0.118	11 ± 1

Selectivity of Binding

The polyamide tethered anthraquinone platinum(II) complexes **2a-c**, preferred to bind to 3'-XXGGXX-5' regions of the DNA without any A/T region selectivity. Whilst it is usual for polyamides with greater than three pyrroles to bind to A/T regions of DNA,[29, 30] it may not be the case for polyamides conjugated to other DNA binders.[31] It appears that the formation of the monofunctional adduct determines the adduct profile.

A possible explanation for the similarity of the binding profiles is that the platinum coordinated to the bases of DNA before the intercalator and the polyamides could direct the complex to AT rich regions of DNA. Platinum binding to nucleobases is kinetically controlled[32, 33] and Davies and co-workers have reported that the initial reaction, formation of monofunctional adducts, determines the adduct profile.[31] This result can be seen in the DNA damage studies as reversible DNA binding moieties such as polyamides and AAQs are not controlling the DNA sequence at which the platinum complex binds. Once the aquation of the platinum complex occurs, the platinum adduct is formed and the extra DNA binding moieties may or may not participate in binding to DNA. The adduct profile reveals that the preference for GG sites of the hybrid platinating complex is similar to that of cisplatin.

In a previous study, 9-aminoacridine carboxamide Pt complexes, containing an intercalating group, were found to have an altered DNA sequence specificity and preferred binding at 5'-CG dinucleotides rather than consecutive guanine nucleotides. The 9-aminoacridine intercalating group had a preference for 5'-CG dinucleotides and directed Pt binding to neighbouring G nucleotides.[19]

Conclusions

DNA damage studies with the platinum complexes using two different plasmids showed that linking polyamides to 1,5C3 tethered platinum(II) complexes did not generate DNA damage profiles that are significantly different to that of cisplatin. This was found not to be the case for the $[1C3\text{-}Pt(II)]^{+}$ complex which lacked the polyamide group. The DNA unwinding studies revealed that the AAQs in the hybrid platinum complexes probably participate in intercalation, but the polyamides in these platinum complexes do not direct the complex to A/T rich regions.

Complexes **2a-c** having one group designed to associate with the minor groove of DNA and one designed to coordinate in the major groove are expected to bind as threading intercalators. Threading requires substantial reorganisation of the DNA and a half-life of approximately 10 hrs has been reported for threading of a 1,5-substituted anthraquinone.[34] This is about thirty-fold slower than the coordinative binding of a platinum acridine complex with a coordination environment almost identical to those of the complexes investigated here.[35] The initial interaction of **2a-c** was expected to be dominated by the association of the polyamide moiety with the minor groove of the DNA because these interactions are expected to be substantially stronger than non-coordinative interactions of the platinum moiety with the major groove. However, the slow rate of threading will allow for equilibration to occur, leading to the kinetic sink of platinum-DNA bond formation dominating the selectivity. Consistent with this is the observation that the polyamide complexes have sequence selectivities that are more similar to that of cisplatin than does Pt-1C3 which is believed to intercalate rapidly.

Thus, it appears that tethering more DNA binding moieties to a platinum complex may not in all cases result in sequence selectivity different from that of cisplatin. In the present case, that is consistent with the disappointing lack of increase in the cytotoxicity of the polyamide-anthraquinone-platinum complexes relative to that of cisplatin.[7] It is likely that longer polyamides would lead to stronger binding to the major groove, potentially allowing this moiety to dominate the sequence selectivity and it is notable that **2c** is the most cytotoxic of the complexes investigated despite a lower level of cellular accumulation.

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