GA and AG Sequences of DNA React with Cisplatin at Comparable Rates

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Abstract: The sequence selectivity of the antitumor drug cisplatin (*cis*-[$PtCl_2(NH_3)_2$] (**1**)) between the 5′-AG-3′ and 5′-GA-3′ sites of DNA has been a matter of discussion for more than twenty years. In this work, we compared the reactivity of GA and AG sequences of DNA towards the aquated forms of cisplatin (*cis*-[$PtCl(NH_3)_2(H_2O)]^+$ (**2**), *cis*-[$Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), and *cis*-[$Pt(OH)(NH_3)_2(H_2O)]^+$ (**4**)) using two sets of experiments. In the first, we investigated a DNA hairpin, whose duplex stem contained a TGAT sequence as the single reactive site, and determined the individual rate constants of platination with **2** and **3** for G and A in acidic solution. The rate constants at 20 °C in 0.1 M $NaClO_4$ at pH 4.5 ± 0.1 were 0.09(4) $M^{-1}s^{-1}$ (G) and 0.11(3) $M^{-1}s^{-1}$ (A) for **2**, and 9.6(1) $M^{-1}s^{-1}$ (G) and 1.7(1) $M^{-1}s^{-1}$ (A) for **3**. These values are similar to those obtained previously for an analogous hairpin that contained a TAGT sequence. The monoadducts formed with **2** by both GA purines are extremely long-lived, partly as a result of the slow hydrolysis of the chloro monoadduct at A, and partly because of the very low chelation rate ($1.4 \times 10^{-5} s^{-1}$ at 20 °C) of the aqua monoadduct on the guanine. In the second set of experiments, we incubated pure or enriched samples of **1**, **2**, **3**, or **4** for 18–64 h at 25 °C with a 19 base pair (bp) DNA duplex, whose radiolabeled top strand contained one GA and one AG sequence as the only reactive sites. Quantification of the number of GA and AG cross-links afforded a ratio of about two in favor of AG, irrespective of the nature of the leaving ligands. These results disagree with a previous NMR spectroscopy study, and indicate that GA sequences of DNA are substantially more susceptible to attack by cisplatin than previously thought.

Keywords: antitumor agents · DNA · kinetics · oligonucleotides · platinum

Introduction

The antitumor drug cisplatin (*cis*-[$PtCl_2(NH_3)_2$] (**1**)) reacts with cellular DNA to give 1,2-GG cross-links as the main final products.[1] When the drug passes the cell membrane and enters the low-chloride milieu of the cytoplasm, the two chloride ligands are successively and reversibly exchanged with water; this gives rise to the reactive aqua species, *cis*-[$PtCl(NH_3)_2(H_2O)]^+$ (**2**), *cis*-[$Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), and *cis*-[$Pt(OH)(NH_3)_2(H_2O)]^+$ (**4**). All the three aqua forms, **2**, **3**, and **4**, can react with DNA. Since **2** is formed first, it has an advantage over **3** and **4**. However, **3** and **4** are one to two orders of magnitude more reactive towards DNA guanines than **2**;[2–4] therefore, either the direct reaction of **2** with DNA, or the hydrolysis of **2** followed by a reaction between **3** or **4** and DNA can occur. Which of the two pathways prevails depends on the reaction conditions and, in particular, on the concentration of DNA and chloride present.[4, 5] In the cell nucleus, the local DNA concentration is very high;[6] this would favor a direct reaction with **2**.

Several research teams have investigated the sequence selectivity that cisplatin displays when binding to DNA. Early pioneering work, which quantified Pt–DNA adducts, came from the groups of Fichtinger-Schepman and Eastman. They incubated DNA with cisplatin or [$PtCl_2$(en)], digested the platinated DNA with exonucleases, separated the digests by high- or fast-performance liquid chromatography (HPLC or FPLC), and analyzed the fractions by diverse methods.[7–9] Both groups found approximately 60–65 % of 1,2-GG adducts and 20–25 % of 1,2-AG adducts, but 1,2-GA adducts were not detected.[9, 10] Later work by Fichtinger-Schepman et al. indicated that the diadduct levels had been exaggerated in the initial assays.[11–13] Other workers mapped the exonuclease III cleavage sites[14, 15] or the stop sites of DNA polymerases[16–18] in order to identify the platinum-binding sites. These studies confirmed that the most frequently

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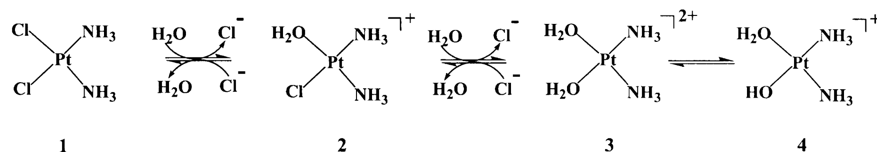
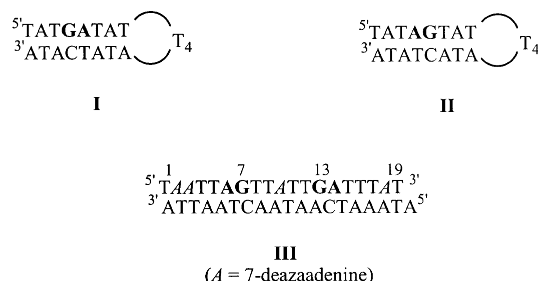


similar hairpin (**II**), which contained the AG site.[4] In addition, we investigated the reactions of the 19 base pair (bp) DNA duplex **III**, in which the radioactively labeled top strand included one AG and one GA reactive site, with solutions containing **1**, **2**, **3**, or **4** as the major species. The number of AG and GA platinum cross-links was determined by using the Maxam – Gilbert sequencing method for G. Thus, the discrimination between GA and AG sites was assessed in an intramolecular competition experiment. All the experiments showed that TAGT is never favored over TGAT by more than a factor of about 2.

platinated sites were G_n ($n \geq 2$) runs, and revealed AG stop sites in some cases.

Combination of polymerase chain reaction (PCR) techniques with the replication mapping procedure afforded a gain in sensitivity[19–25] and enabled the identification of platinum binding sites with a precision of ± 1 nucleotide.[24] In this manner, Murray et al. found that platination levels at GA and AG sequences in plasmid DNA,[19, 22, 23] purified genomic DNA,[20, 24] and intact cells[20, 24, 25] were comparable; this raised the question whether a binding preference for AG really exists.

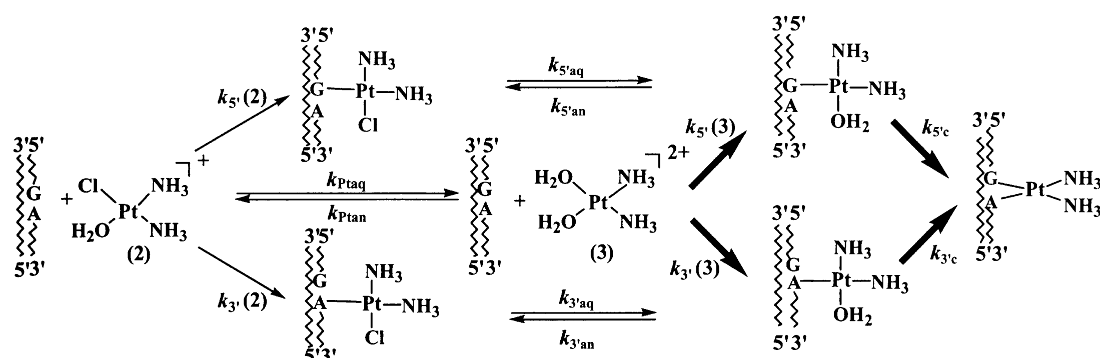
Previously, we have used hairpin DNA oligonucleotides containing GG, AG, GC, and GGC binding sites in the duplex stem to determine the individual rate constants for each guanine or adenine reacting with **2**, **3**, or **4**.[26, 27] We used purified **2** or **3** to start the reaction (instead of generating **2** in situ by hydrolysis of cisplatin), and in the experiments with **2**, we included its reversible hydrolysis to **3** in the data analysis. The hydrolysis of **2** to give **3** is an important pathway at 10^{-4} M concentrations of the DNA reactive sites,[4] unless the chloride concentration exceeds that of the DNA reactive sites by more than a factor of about ten.[28] In the present work, we have determined the individual rate constants, in acidic solution, for the reaction of **2** and **3** with a GA sequence embedded in hairpin **I**. The results were compared to those obtained for a



Results

Stability of hairpin I: We have shown previously that oligonucleotide **II** assumes a hairpin form under our experimental conditions,[26] and the melting curve of a 10^{-4} M solution of **I** in 0.1 M $NaClO_4$ is similar to that of **II**. Oligonucleotide **I** melts at 45 °C, while **II** melts at 46.5 °C,[26] which is well above the experimental temperature (20 °C).

Reaction between hairpin I and the aquated cisplatin forms 2 and 3: The reaction between **I** and **2** in acidic solution is depicted in Scheme 1. Chloride anation of **2** to give **1** could be neglected, because the concentration of chloride (released by hydrolysis of **2** and its monoadducts) at no point in the reaction is high enough to make the anation of **2** significant. The reaction between **I** and **3** corresponds to the subsystem indicated with bold arrows. As previously described for the reactions of **II**,[4] we firstly investigated this subsystem separately, using a chloride-free medium and solid *cis*-$[Pt(NO_3)_2(NH_3)_2]$ as a source of **3**. We then treated **I** with **2** and determined the concentration curves for the unreacted oligonucleotide, the final chelate, and the sums of the chloro and aqua monoadducts at both G and A (Figure 1). These concentration curves were used to optimize the rate constants $k_{5'}(\mathbf{2})$, $k_{3'}(\mathbf{2})$, $k_{5'aq}$, and $k_{3'aq}$ (Table 1). The rate constants $k_{5'}(\mathbf{2})$ and $k_{3'}(\mathbf{2})$ bear an additional error of approximately 20 %, because the concentration of the reactants was determined spectrophotometrically, and is, therefore, imprecise. This error was added to the standard deviations and is included



Scheme 1.

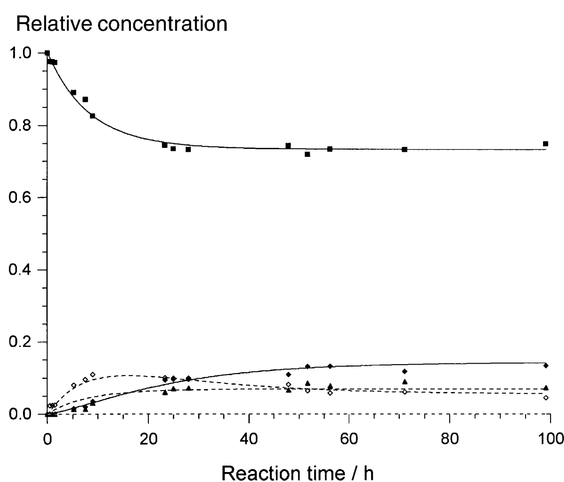


Figure 1. Experimental and calculated concentration curves for one run of the reaction between **2** (ca. 0.025 mM) and **I** (ca. 0.1 mM) in 0.1M $NaClO_4$ at 20 °C and pH 4.5 ± 0.1. **I**, ■ and ——; G-bound monoadducts (chloro and aqua), ◇ and – – –; A-bound monoadducts (chloro and aqua), ▲ and – – –; final chelate, ◆ and ——.

Table 1. Optimized rate constants for the reactions between the complexes **2** and **3**, and the oligonucleotides **I** and **II**[a] (standard deviations in parentheses include errors in concentration measurements).

	Platination [$M^{-1}s^{-1}$]			
	$k_{5'}$(**2**)	$k_{3'}$(**2**)	$k_{5'}$(**3**)	$k_{3'}$(**3**)
5'TAT**GA**TAT / 3'ATACTATA T4 (**I**)	0.09(4)	0.11(3)	9.6(5)	1.7(1)
5'TAT**AG**TAT / 3'ATATCATA T4 (**II**)	0.08(3)	0.19(6)	1.5(3)	9.0(10)
	Aquation [10^{-5} s^{-1}]		Chelation [10^{-3} s^{-1}]	
	$k_{5'aq}$	$k_{3'aq}$	$k_{5'c}$	$k_{3'c}$
5'TAT**GA**TAT / 3'ATACTATA T4 (**I**)	≤ 0.3	≤ 0.05	0.014(1)	1.3(1)
5'TAT**AG**TAT / 3'ATATCATA T4 (**II**)	0.26(6)	0.65(2)	0.3(3)	0.08(1)

[a] Reference [4].

in the values given in Table 1. (This error was not taken into account in Table 1 of ref. [4].)

The rate constants for platination, aquation of the chloro monoadducts, and chelation of the aqua monoadducts, together with those determined previously for hairpin **II**, are listed in Table 1.[4] The rate constants for platination of **I** with **3** are, within the error limits, identical to those found for **II** (this is true for both adenine and guanine). The global platination rate constants for the AG and GA sequences are approximately three times smaller than those for the GG sequence.[4] The individual rate constants for **3** are greater than for **2**; by a factor of approximately 15 for adenine, and between 50 and 150 and for guanine. Thus, our data show the expected trends and are internally consistent. Comparison of the rate constants obtained for the reaction of **2** with **I** (sequence GA) to those with **II** (sequence AG) indicates that the values for adenine are similar, whereas the rate constant for guanine of GA is about half as large as that of AG. The differential decrease of the individual rate constants when going from **3** to **2** could be due to two effects. Firstly, the relative accessibility, which our previous analysis indicated to be a principal factor in determining the relative reactivity of a given base,[29] depends on the size of the attacking species, and the sizes of **2** and **3** are likely to be different. Secondly, the 6-amino group of adenine could form a hydrogen bond to the chloride of **2** in the transition state; this could explain why the rate constants for adenine decrease to a lesser extent than for guanine.

The very small chelation rate constant of 0.000014 s^{-1} observed for the aqua monoadduct formed on the guanine of GA indicates that this adduct has a half-life of approximately 14 h at 20 °C. Steric factors, which seem to disfavor chelation of a monoadduct by a 3′-purine (see the slow chelation of the 5′-monoadducts at GG and AG sequences[4]), and the inherently lower reactivity of adenine relative to guanine could account for the slow chelation.

The rate constants for aquation of the monoadducts could not be determined with precision. However, we were able to determine the upper limits (Table 1), which indicated that aquation of both GA monoadducts is markedly slower than that of *cis*-$[PtCl(NH_3)_2(dGuo)]^+$ (1.4×10^{-5} s^{-1}).[30] Although slow aquation of monoadducts has been observed for the AG sequence (Table 1) and the 5′-G of GG,[4] the aquation of the GA monoadduct adenine ($k_{3'aq} \leq 5 \times 10^{-7}$ s^{-1}) is the slowest ever observed for a chloro monoadduct of cisplatin. Unfortunately, reference data is not available for the rates of hydrolysis of platinum–chloro complexes bound to adenine. It is possible that hydrolysis of chloro complexes with a *cis*-adenine is impeded relative to that of analogous guanine complexes, because the chloro ligand can accept a hydrogen bond from the NH_2 group of adenine (as observed, e.g., in the crystal structure of $[PtCl_3(9\text{-methyladeninium})]$[31]), which, in turn, would stabilize the chloro form of the adenine complex. Similarly, the aqua ligand can donate a hydrogen bond to the O6 atom of guanine.

Reaction between duplex III and the various forms of cisplatin: Three sets of reactions between duplex **III** and either form of cisplatin were performed. The first two sets were run in $NaClO_4$ without the addition of buffer and were heated at 25 °C for 18 and 64 hours, respectively. In each set the duplex was reacted with: 1) freshly dissolved cisplatin; 2) a 0.01M solution of cisplatin, which had been stirred for 24 h with 1 equivalent of $AgNO_3$; and 3) a 0.01M solution of cisplatin, which had been stirred for 24 h with 2 equivalents of $AgNO_3$. The final mixtures (pH 3.8) were approximately 0.2 mM in the duplex, 0.1 mM in platinum, and 0.1M in $NaClO_4$. Solutions 1) and 3) contained only **1** and **3**, respectively, while solution 2) was enriched with **2** and contained smaller amounts of both **1** and **3**.[32] At pH 3.8, only 2.6% of **3** dissociates to give **4** ($pK_a = 5.37$),[33] therefore, the consumption of **3** during the reaction with DNA should not significantly affect the pH. The third set (carried out only with **1** and **3**) contained a 0.01M phosphate buffer (pH 7) and was incubated for 18 hours at 25 °C. At this pH the equilibrium between **3** and **4** lies completely on the side of **4**. The top strand, which contained only the AG and GA sequences as

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reactive sites (all the adenines of the top strand were replaced by 7-deazaadenine), was radioactively labeled, whereas the bottom strand was unlabeled. Initial 20% polyacrylamide gel electrophoresis (PAGE), allowed the fraction containing the top strand covalently bound to one equivalent of platinum to be separated from the unplatinated and diplatinated top strands (Figure 2). The band with the monoplatinated top

Figure 2. Fractionation by 20% denaturing PAGE of the products of the reactions between **III** and **1**, **2**, or **3**, according to the number (0, 1, or 2) of equivalents of platinum bound to the top strand of **III**. Only the top strand was radiolabeled. Unreacted **III** was used as a control (left lane). The reactants were incubated in 0.1M $NaClO_4$ for 18 h at 25 °C and pH 3.8.

strand was cut from the gel and analyzed by the Maxam–Gilbert sequencing reaction for guanine (Figure 3).[34] Since platinated guanines do not react with dimethyl sulfate,[35] the decrease in intensity of the band corresponding to a given

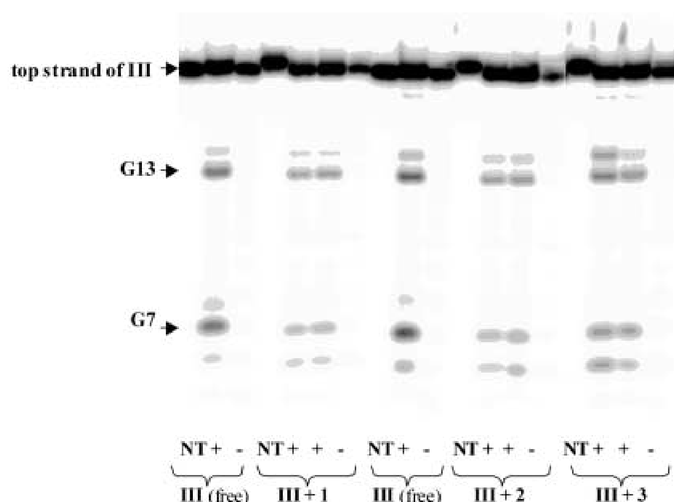

Figure 3. Analysis of the monoplatinated top strand fraction from the reaction between **III** and **1**, **2**, or **3** using 20% denaturing PAGE. The reactants were incubated in 0.1M $NaClO_4$ for 18 h at 25 °C and pH 3.8. The unreacted top strand of **III** was used as a control. NT: non-treated sample; "+": DMS/piperidine followed by NaCN treatment; "–": piperidine alone followed by NaCN treatment.

guanine residue (with respect to the unplatinated top strand) can be used to calculate the percentage of platination at this site. The results are summarized in Table 2. They show that the AG/GA platination ratio varies between 1.5 and 2.8. There is no apparent correlation between the AG/GA ratios and the incubation conditions or the type of platinum complex used. Thus, the differences probably reflect experimental errors. We conclude that the discrimination between the AG and GA sites of **III** is approximately 2, irrespective of the pH or the nature of the platinating species.

Table 2. Relative inhibition of DMS/piperidine cleavage [%] by the binding of cisplatin and its aqua forms to **III**, and 3′-exonuclease stops at the AG and GA sites.

Incubation conditions	Complex	A**G** cleavage[a]	A**G** 3′-exo stop	**G**A cleavage[a]	**G**A 3′-exo stop	A**G**/**G**A
18 h 25 °C	**1**	62	G7	29	A14	2.1
pH 3.8	**2**	65	nd[b]	25	nd[b]	2.6
0.1M $NaClO_4$	**3**	56	G7	35	A14/G13	1.6
64 h 25 °C	**1**	61	G7	27	A14	2.2
pH 3.8	**2**	68	G7	35	A14	1.9
0.1M $NaClO_4$	**3**	56	G7	20	A14	2.8
18 h 25 °C	**1**	59	G7	22	A14	2.7
pH 7.0	**4**	55	G7	36	A14	1.5
0.01M phosphate						

[a] % cleavage intensity with respect to that observed for the unplatinated **III** at the same site. [b] nd = not determined.

The Maxam–Gilbert sequencing method cannot, a priori, distinguish between a monoadduct at G and a diadduct (AG or GA chelate). According to Figure 1, a significant proportion of monoadducts could still be present for GA after 18 h at 25 °C (corresponding to about 25 h at 20 °C), but after 64 h at 25 °C (corresponding to about 90 h at 20 °C), this fraction should be considerably smaller. For AG, the proportion of monoadducts should be much smaller after 18 h, and insignificant after 64 h.[4] As the monoadducts on the adenine of GA could not be quantified by our method, the AG/GA ratio given in Table 2 may have been overestimated. However, the presence of a significant amount of monoadducts is unlikely, because between 18 and 64 h of incubation neither the proportion of platinated species, nor the AG/GA ratio changed. Furthermore, the percentages of cleavage inhibition at AG and GA sites (Table 2) add up to, independently of the incubation time, a value close to 100% (since the two values are determined separately, their sum can exceed 100%), which suggests that the amount of monoadducts was indeed negligible. Moreover, digestion of the platinated strand with 3′-exonuclease stopped almost exclusively at G7 and A14 (Table 2), showing that the platination of G13 seen in the Maxam–Gilbert sequencing arises from a G13–A14 cross-link.[36] All these observations indicate that the values given in the last column of Table 2 correspond to the ratio of intrastrand AG–Pt and GA–Pt cross-links.

It is conceivable that in the denaturing, chloride-free conditions favorable for intrastrand chelation,[37] which were used in the separation of the monoplatinated species (Figure 2), most of the remaining monoadducts formed the intrastrand cross-link.

Discussion

The present results suggest that TGAT and TAGT sequences of duplex DNA are platinated with comparable rates. Complex **3** does not significantly discriminate between the GA and AG groups of hairpins **I** and **II**, and displays approximately a two-fold preference for AG over GA upon reaction with duplex **III**. Complex **2** reacts with the AG guanine of **I** about two times faster than with the GA guanine of **II** (as judged

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from the average rate constants shown in Table 1); however, global platination rates for the whole dinucleotide are, within experimental error, equal. For the reaction of enriched **2** with **III** at pH 3.8, the final ratio (about 2) observed between the AG and GA cross-links does not reflect the selectivity of **2** alone since: 1) the initial solution contains a substantial amount of **3**;[32] and 2) a portion of **2** reacts with **III** by hydrolysis to give **3**. Nevertheless, since pure **3** favors AG over GA by approximately 2:1 (vide supra), we can conclude that the net discrimination between AG and GA for **2** is also close to 2. The aqua–hydroxo species **4**, generated from **3** at pH 7, shows an AG/GA discrimination of about 1.5. Finally, reactions with freshly dissolved **1**, which proceed by successive hydrolysis to give **2**, and either **3** (pH 3.8) or **4** (pH 7), also yielded, independently of the incubation conditions, AG/GA ratios of about 2.

We would like to caution that the platination ratio of about 2:1 was determined for AG and GA sites flanked by T residues, and cannot be directly compared with the number of AG–Pt and GA–Pt crosslinks obtained from platination of random DNA. Our results nevertheless clearly indicate that GA sites of duplex DNA are attacked by cisplatin to a significant extent.

Our finding that the reactivity of TAGT and TGAT sites towards **2** differs at most by a factor of about two contrasts with the recent NMR spectroscopy study reported by Hambley, Berners-Price et al., in which a 20–50-fold discrimination was found, depending on the data treatment.[5, 38] The discrepancy between their data and ours (Table 1) prompted us to investigate the intramolecular competition between AG and GA groups embedded in similar local environments within duplex **III**, whereby potential errors originating from a comparison of the results of two different experiments would be eliminated. In addition, we also examined the effects that changes in pH and incubation time had on the balance between AG–Pt and GA–Pt cross-links. Our findings confirmed the results obtained for hairpins **I** and **II**. The reasons for the disagreement between our results and those of Hambley, Berners-Price et al. are not yet clear. However, it should be noted that Hambley et al. used the same methodology to investigate the reactions of AG and GA containing DNA duplexes with the diaqua complex **3**,[38] and reported similar platination rates for GA and AG sites, in contrast to the large difference they observed with the complex **2**. Since we have recently found that the sequence dependence of platination rates for **3** can be explained on the grounds of steric and electrostatic factors,[29] it appears unlikely that **2** and **3** would show such different selectivities. Our present results, on the other hand, which indicate at most a twofold TAGT over TGAT discrimination both for **2** and **3**, agree with our kinetic model.[29] We wish to point out that the 7-deazaadenines of the top strand of **III** were all at least three nucleotides away from the AG and GA sequences; therefore, the contribution of these "unnatural" bases to the electrostatic potential at the reacting sites was negligible.

In light of our results, one may wonder why no GA cross-links were found in a number of previous studies that quantified cisplatin–DNA adducts.[7–10, 13, 18, 39–42] Firstly, this could be due to the extremely long-lived GA monoadducts (Figure 1); as a result of the monoadducts slowly converting into diadducts, a proportion of the GA adducts might have been quantified as monoadducts in some of the assays. Secondly, GA adducts were never specifically targeted. Workers quantifying digested DNA fragments by HPLC or FPLC usually verified the identity of the Pt–AG fraction either by NMR spectroscopy,[9] digestion and subsequent deplatination,[10] co-elution with an authentic dinucleotide (p)ApG cross-linked with the platinum complex,[40, 42] or by using monoclonal antibodies raised against *cis*-$[Pt(NH_3)_2(pApG)]$.[13, 39, 43] Unfortunately, checks with authentic samples of *cis*-$[Pt(NH_3)_2(pGpA)]$ or with antibodies raised against this species were not carried out. Therefore, GA cross-links may have escaped detection. Thirdly, exonucleases and polymerases may bypass the GA diadducts more efficiently than GG and AG diadducts; however, we believe that this is unlikely. We have shown in this study that the 3′-exonuclease phosphodiesterase I from *crotalus adamanteus* stops at both AG and GA cross-links. As far as DNA polymerases are concerned, Comess et al. have shown that some polymerases do bypass GG and AG platinum cross-links,[44] but the differences (up to 16%) were not sufficient to prevent a particular kind of cross-link from being detected. Lastly, the exonuclease and polymerase mapping experiments, which used only one cycle of primer extension,[16–18] were not as sensitive as the later PCR-based methods;[19–25] and the fraction of GA cross-links present may have been below the detection limit. In addition, heat used for denaturation during the PCR cycles may have contributed to a complete conversion of the monoadducts to diadducts.

The fact that GA sequences of DNA are significantly attacked by cisplatin raises the question whether cisplatin–GA adducts could be of biological importance. Individual GG–Pt and AG–Pt intrastrand cross-links have been shown to have differential cytotoxic and mutagenic effects in bacteria.[45–47] The biological effects of GA–Pt corsslinks may be again different. Unfortunately, since the GA–Pt crosslinks have so far been considered as nonexistent, their effects in vivo have never been tested. The fact that GA monoadducts are long-lived (Figure 1) means that they may have a biological role of their own, for instance, by covalently trapping repair proteins[48] or other cellular factors. We believe that the cellular processing of Pt–DNA adducts on GA sequences merits further investigation.

Experimental Section

Starting materials: The oligonucleotides were synthesized as ammonium salts by the group of T. Huynh-Dinh (Institut Pasteur, Paris) (**I**) and by Eurogentec, Inc. (**III**). The purity of each compound was checked by HPLC or by gel electrophoresis. Approximate concentrations were evaluated photometrically by using an average molar absorption coefficient (ε_{260nm}) of 8000 $M^{-1}cm^{-1}$ per nucleotide. Cisplatin was kindly provided by Johnson–Matthey. For the kinetic experiments with **I**, the solutions of *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**)[49] were prepared as described previously. Solutions of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) were prepared by dissolving *cis*-$[Pt(NO_3)_2(NH_3)_2]$[50] in water. For the reaction with **III**, the solutions of **2** and **3** were prepared by stirring cisplatin for 24 h at ambient temperature with 1 and 1.99 equivalents of $AgNO_3$, respectively. Solutions of cisplatin were prepared either from a powder (0.05 mmol), which was sonicated with

H_2O (0.5 mL) for 15 min, or by dissolving the powder in dimethyl sulfoxide (DMSO) (50 μL) and diluting the resultant solution with H_2O (450 μL) (The complexes were diluted a further 100 times in the reaction mixtures, therefore, the final DMSO concentration was 0.1 %.) The preparation with DMSO was chosen to match the solution composition used in the initial work by Fichtinger-Schepman et al.[7]. T4-Polynucleotide kinase and [γ^{32}P]-adenosine 5′-triphosphate (ATP) were purchased from Pharmacia Biotech, dimethylsulfate (DMS) and piperidine were bought from Sigma, and NaCN was supplied by Merck.

Kinetic analysis of the reactions of I with 2 and 3: The reactions were performed in 0.1 M $NaClO_4$ at pH 4.5 ± 0.1, as described previously for **II**.[4] To avoid diplatination of the oligonucleotide and formation of hydroxide dimers, the reaction solutions were approximately 0.1 mM in **I** and 0.02–0.08 mM in **2** or **3**. HPLC analysis and calculation of the rate constants was conducted in the same manner as described for **II**,[4] except that for the data analysis we employed the SCIENTIST program.[55] In Scheme 1, the rate constants k_{Ptaq} (1.8×10^{-5} s^{-1}) and k_{Ptan} (6.43×10^{-2} M^{-1}s^{-1}), as well as $k_{5'an}$ and $k_{3'an}$ (7.5×10^{-2} M^{-1}s^{-1}) were kept fixed.[51] The reaction system that involved **3** (bold arrows in Scheme 1) was first investigated separately in chloride-free solution and the rate constants $k_{5'}$(**3**), $k_{3'}$(**3**), $k_{5'c}$, and $k_{3'c}$ were determined. The reaction between **I** and **2** was then examined, and the concentration curves for the starting oligonucleotide, the final chelate, and for the sums of chloro and aqua monoadducts for 5′ and 3′ were determined. These last were used to optimize the rate constants $k_{5'}$(**2**), $k_{3'}$(**2**), $k_{5'aq}$, and $k_{3'aq}$, while keeping the previously determined values for $k_{5'}$(**3**), $k_{3'}$(**3**), $k_{5'c}$, and $k_{3'c}$ fixed. The values given in Table 2 are weighted averages over at least two experiments.

Reactions of III: The top strand of **III** was 5′-end-radiolabeled by using polynucleotide kinase and [γ^{32}P]ATP, and was purified using 20 % PAGE. The 5′-end-radiolabeled top strand of **III** was then mixed with an excess of non-radiolabeled material and annealed with the bottom strand either in 0.11 M $NaClO_4$ acidified by $HClO_4$, or in 0.011 M phosphate buffer (pH 7). The solution containing **III** (18 μL, ca. 0.22 mM) was then mixed with a 1 mM solution of the platinum complex (2 μL). The final reaction mixtures were approximately 0.2 mM in **III**, 0.1 mM in platinum, and 0.1 M in $NaClO_4$, and had either a pH of 3.8 ($NaClO_4$) or 7 (0.01 M phosphate buffer). The reaction mixtures were incubated for 18 or 64 h at 25 °C.

The platinated oligonucleotides were separated by 20 % denaturing PAGE [7 M urea, 0.088 M Tris-Borate pH 8.3, 2 mM ethylenediaminetetraacetic acid (EDTA)] at room temperature. After elution from the gel and ethanol precipitation, the material from each band was treated with DMS and piperidine, under Maxam–Gilbert sequencing conditions,[35, 52] in order to determine the amount of oligonucleotide platinated on G7 or G13. The oligonucleotides were then deplatinated by being heated with 1 M NaCN for 18 h at 37 °C, and the subsequent products were precipitated. After migration on a 20 % polyacrylamide gel, the various spots were quantified by means of a dynamic molecular phosphorimager, and the data was processed with Imagequant software. As the N7-platinated guanines were no longer reactive with DMS, the relative platination rates could be deduced from the reduced intensity of the spots which corresponded to cleavage at G7 and G13.[34]

In order to determine the platinum-binding sites, the PAGE band which contained the top strand complexed with 1 equivalent of platinum was examined by 3′-exonuclease digestion, because 3′-exonuclease stops at platinum monoadducts and chelates.[53] The monoplatinated products were incubated in 10 mM Tris-HCl buffer pH 8.0 in the presence of $MgCl_2$ (2 mM) and *t*RNA (0.5 mg mL^{-1}), with the 3′-exonuclease phosphodiesterase I, derived from *crotalus adamanteus* venom (0.023–0.046 unit mL^{-1}, Worthington Biochemical Corporation), for 30 min at 37 °C. The digested mixture was run on a 20 % denaturing gel to afford two major fragments. Each of them was eluted from the gel, precipitated, and deplatinated by 1 M NaCN for 18 h at 37 °C. After precipitation, the deplatinated fragments were migrated on 20 % denaturing gel, and their migrations were compared to the migrations of the fragments that were obtained by partial digestion of the starting oligonucleotide with 3′-exonuclease at 0.0015 unit mL^{-1}.

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