A Complete Kinetic Study of GG versus AG Platination Suggests That the Doubly Aquated Derivatives of Cisplatin Are the Actual DNA Binding Species

Franck Legendre, Véronique Bas, Jiří Kozelka,* and Jean-Claude Chottard*[a]

Abstract: The hairpin-stabilized double-stranded oligonucleotides d(TAT*GG*-$TATT_4ATACCATA$) (**I**) and d(TAT*AG*-$TATT_4ATACTATA$) (**II**) were allowed to react with the three aquated forms of the antitumor drug cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, **1**) which are likely candidates for DNA binding, that is, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**), *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**), and its conjugate base *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ (**4**). The reaction between **I** and $[Pt(NH_3)_3(H_2O)]^{2+}$ (**5**) was also studied for comparison. All reactions were monitored by HPLC. The platination reactions of **I** and **II** were carried out in $NaClO_4$ (0.1M) at 293 K and at a constant pH of 4.5 ± 0.1 for **2**, **3**, and **5**. The data relative to the platination by **4** were obtained from measurements in unbuffered $NaClO_4$ solutions (0.1M) at a starting pH close to neutrality, where **3** and **4** are present in equilibrium. In this case, a fit function describing the pH–time curve allowed the determination of the actual concentrations of **3**, **4**, and the dihydroxo complex. The platination rate constants characterizing the bimolecular reactions between either **I** or **II** and **2**, **3**, and **4** were individually determined along with the rate constants for hydrolysis of the chloro-monoadducts and for the chelation reactions of the aqua-monoadducts. The reactivity of compounds **2**–**5**, which have the general formula *cis*-$[Pt(NH_3)_2(H_2O)(Y)]^{2+/+}$, decreases in the order $\mathbf{3} > \mathbf{4} > \mathbf{5} >> \mathbf{2}$, that is, $Y = H_2O > OH^- > NH_3 >> Cl^-$, which is the order of decreasing hydrogen-bond donating ability of Y. Deprotonation of **3** to **4** reduces the reactivity of the platinum complex only by a factor of ≈ 2, and both complexes discriminate between the different purines of **I** and **II** in the same manner. Whereas **3** and **4** react approximately three times faster with the GG sequence of **I** than with the AG sequence of **II**, **2** shows a similar reactivity towards both sequences. In view of the well-established preferential binding of cisplatin to GG sequences of DNA in vivo and in vitro, this result suggests that the actual DNA platination species are derived from double hydrolysis of cisplatin.

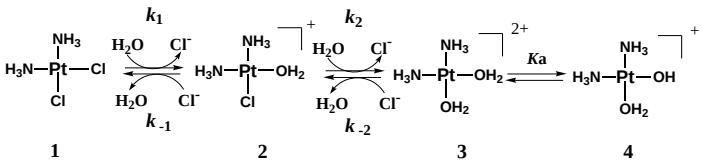
Keywords: antitumor agents · kinetics · oligonucleotides · platinum complexes

Introduction

The antitumor drug cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, **1**) reacts with cellular DNA, primarily binding to guanine bases.[1–5] This reaction is sequence-selective, and G_n sequences ($n \geq 2$) represent major sites for platinum binding. Thus, administration of cisplatin to cells in culture, intact tissues, or to the blood serum of cancer patients, leads to an over statistical formation of intrastrand *cis*-$Pt(NH_3)_2^{2+}$ chelates between adjacent guanines ("GG–Pt crosslinks"), amounting to 60–65% of all platinum bound to DNA. The second most abundant adducts are intrastrand AG*cis*-$Pt(NH_3)_2$ chelates ("AG–Pt crosslinks"), which account for approximately 20% of the platinum bound, whereas, interestingly, GA crosslinks ("GA–Pt crosslinks") are formed only to a minor extent and could not be quantified.[6–10]

This sequence-selective cisplatin binding to DNA has prompted a number of recent studies. Several groups including ours have investigated the kinetics of reactions between oligonucleotides and different cisplatin forms present in aqueous solution, that is, *cis*-$[PtCl_2(NH_3)_2]$ (**1**), *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**), and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) (see Scheme 1).[11–18]

The rate-determining step of the reaction between **1** and DNA has been shown to be the hydrolysis of **1** to **2**.[19–21]



Scheme 1.

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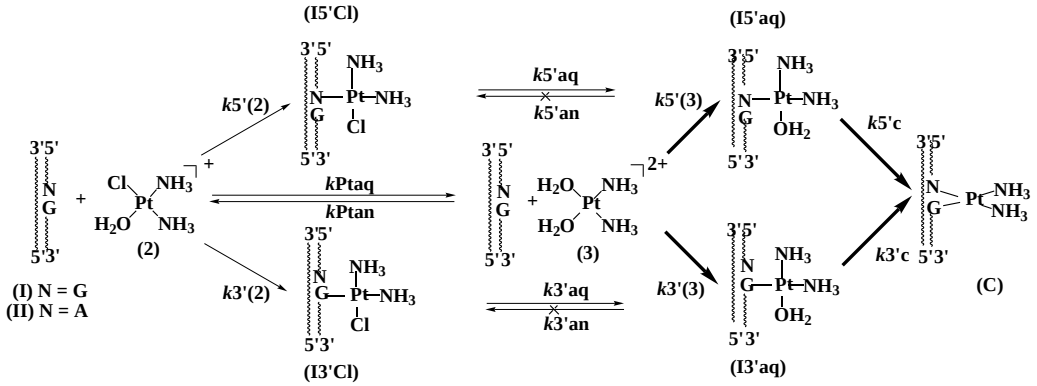
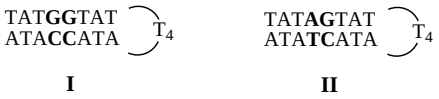


Scheme 2.

Depending on the reaction conditions, **2** was found to react with DNA bases either directly, or by means of hydrolysis to **3**/**4**.[22] Which pathway dominates the DNA platination in vivo, has not yet been conclusively demonstrated. We have recently reported that the diaqua form **3** reacts with the GG and AG sequences embedded in the identical environments of the two hairpin-stabilized duplexes **I** and **II**, respectively, with an overall rate ratio of 3:1.[18] Since this ratio coincides with that of the relative amounts of the GG – Pt and AG – Pt crosslinks

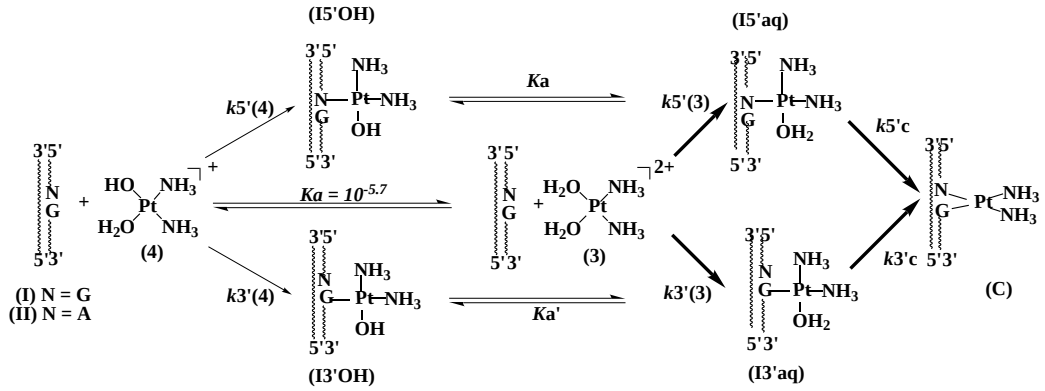


found after DNA treatment with cisplatin in vivo, our finding suggested that **3** might be the cisplatin form which interacts with DNA. However, the binding selectivities of the monochloro-monoaqua form **2** and of the conjugate base of **3**, *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ (**4**) remained to be determined.

To evaluate the individual platination rate constants specific for **2** and for **4**, several experimental difficulties had to be surmounted. Reactions of **2** in acidic conditions are complicated by the hydrolysis of **2** to **3** (Scheme 2) and this occurs in parallel with the direct reaction of **2** with the oligonucleotide. Since **3** is considerably more reactive towards nucleobases than **2**,[22, 23] the commonly used approximation neglecting the reaction between **3** and the oligonucleotide[12, 13, 16, 24] yields unprecise results. Our solution to the problem resided in separate investigations of the reaction of **3** in a series of experiments that we reported earlier[18] (indicated by bold arrows in Schemes 2 and 3) and allowed the reactions of **2** (Scheme 2) and **4** (Scheme 3) to be studied with a reduced number of variables.

The problem which has so far impeded the measurement of the rate constants relevant to the aquahydroxo species **4** is that the pK_a defining the **3**/**4** equilibrium (5.37[25]) is relatively close to that characterizing the deprotonation of **4** (7.21[25]), therefore at no pH can **4** be the only protolytic form of the diaqua complex of cisplatin. Moreover, maintaining a constant pH between 6 and 7 is extremely difficult without using a buffer. The reactions of **4** with **I** or **II** were, therefore, first investigated with **3** in acidic solution (at constant pH), and subsequently in a solution which was adjusted at the beginning to neutral pH and then monitored without readjustments. The pH – time curve was entered into the program calculating the theoretical concentration curves of the various species in the form of a fit function as described previously.[26] The protolytic equilibria between **3**, **4**, and *cis*-$[Pt(OH)_2(NH_3)_2]$, as well as those for the aqua-monoadducts, were explicitly modeled, as described[26] (Scheme 3).

Abstract in French: *Les réactions entre les oligonucléotides d(TATGGTATT$_4$ATACCATA) (**I**) et d(TATAGTATT$_4$ATACTATA) (**II**), dont la double hélice est stabilisée par formation d'une épingle à cheveux, et les trois dérivés aqua du cisplatine (cis-$[PtCl_2(NH_3)_2]$, **1**) suscepibles de se coordiner à l'ADN in vivo, cis-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**), cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**3**) et sa base conjuguée cis-$[Pt(OH)(NH_3)_2(H_2O)]^+$ (**4**) ont été étudiées par HPLC. La réaction entre **I** et $[Pt(NH_3)_3(H_2O)]^{2+}$ (**5**) a été analysée à titre de comparaison. Les réactions de **I** et **II** avec **2**, **3** et **5** ont été réalisées en solution aqueuse 0,1M en $NaClO_4$ à 293 K et à pH constant 4,5 ± 0,1. Les résultats relatifs aux platinations par **4** ont été obtenus en milieu non tamponné, $NaClO_4$ 0,1M, en partant d'un pH proche de la neutralité auquel **3** et **4** sont en équilibre. Dans ces cas, une fonction dâjustement décrivant la variation du pH avec le temps a permis de déterminer les concentrations de **3** et **4** et du complexe dihydroxo cis-$[Pt(OH)_2(NH_3)_2]$. Les constantes de vitesse de platination bimoléculaire entre **I** ou **II** et **2**, **3** et **4** ont été déterminées individuellement ainsi que celles d'hydrolyse des monoadduits-chloro et de chélation des monoadduits-aqua. La réactivité des complexes **2**–**5** de formule générale cis-$[Pt(NH_3)_2(H_2O)(Y)]^{2+/+}$ décroît dans l'ordre **3** > **4** > **5** >> **2**, soit $Y = H_2O > OH^- > NH_3 >> Cl^-$, qui correspond à l'aptitude décroissante de Y comme donneur de liaison hydrogène. La déprotonation de **3** en **4** réduit la réactivité du complexe seulement d'un facteur 2 et **3** et **4** présentent les mêmes sélectivités de liaison vis-à-vis de **I** ou **II**. Alors que **3** et **4** réagissent environ trois fois plus vite avec la séquence GG de **I** comparée à AG de **II**, le complexe **2** présente la même réactivité pour les deux séquences. Au regard de la liaison préférentielle, bien établie, du cisplatine sur les séquences GG de l'DN, in vitro comme in vivo, nos résultats suggèrent que les entités se coordinant effectivement à l'ADN soient dérivées de la double hydrolyse du cisplatine.*



Scheme 3.

One specific objective of this work was to determine the influence of the platinum ligands on the kinetics and selectivity of DNA binding. For the sake of comparison, we include the case of the reaction of **I** with $[Pt(NH_3)_3(H_2O)]^{2+}$ (**5**). A second aim was the evaluation of the influence of the base sequence on platination, aquation of the chloro-monoadducts, and chelation of the aqua-monoadducts.

Results and Discussion

Stability of the hairpin structures

We have shown previously that the hairpins **I** and **II** assume the monomolecular duplex structure under our experimental conditions, and their melting temperatures were determined as 55 and 46.5 °C, respectively, well above the experimental temperature of 20 °C.[18] Local conformational changes induced by *cis*-$Pt(NH_3)_2(Y)$ moieties ($Y = NH_3$, Cl^-) bound to duplex oligonucleotides appear to depend on the base sequence[27] and were undetectable by CD in the case of $d(TTGGCCAA)_2$[15] as well as with duplex DNA.[28] We can thus assume that the monoadducts of **I** and **II** remain in the duplex-hairpin form. The chelation of *cis*-$Pt(NH_3)_2^{2+}$ by two adjacent purines is known to distort the duplex structure;[29] in the case of **I**, the GG *cis*-$Pt(NH_3)_2$ diadduct decreases the melting temperature in $NaClO_4$ solution (0.1 M, $pH = 4.5 \pm 0.1$) from 55 to 43 °C. No T_m measurement was carried out for the diadduct of **II**; partial disruption of the duplex structure is expected at 20 °C. Since the formation of both the mono and diadducts (Schemes 2 and 3) is irreversible under our experimental conditions, the duplex distorsion after the chelation steps is not relevant to the kinetic analysis.

Methodological considerations

The main obstacle which complicates the analysis of the reactions between the monoaqua-monochloro complex **2** and oligonucleotides is the fact that **2** can react either directly, to form chloro-monoadducts, or through solvolysis to **3**, to yield aqua-monoadducts (Scheme 2). In this work, we took both pathways explicitly into account. Initially we examined the reaction system which involved the platination of **I** or **II** by **3** in the absence of chloride[18] (bold arrows in Scheme 2), at a pH maintained between 4.4 and 4.6. Subsequently, the reactions between approximately stoichiometric amounts of the oligonucleotides and **2** (isolated according to ref.[30]) were monitored. In these runs, we quantified the concentration of the unreacted oligonucleotide, that of the final chelate [**C**], and the sums of the monoadducts concentrations $[\mathbf{I5'}] = [\mathbf{I5'Cl}] + [\mathbf{I5'aq}]$ and $[\mathbf{I3'}] = [\mathbf{I3'Cl}] + [\mathbf{I3'aq}]$ (Scheme 2). The differential equations used for the kinetic analysis took into account all the reactions shown in Scheme 2 except the chloride anations characterized by the rate constants $k_{5'an}$ and $k_{3'an}$. The latter could be neglected, since the maximum chloride concentration (0.1 mM) was low enough to warrant that the values of the products $k_{5'an}[Cl^-]$ and $k_{3'an}[Cl^-]$ were inferior to $k_{5'c}$ and $k_{3'c}$ by more than one order of magnitude at any time, for any reasonable anation rate constants $k_{5'an}$ and $k_{3'an}$.[31] Numerical integration of the differential equations and fits to the experimental curves (see Figure 1 for an

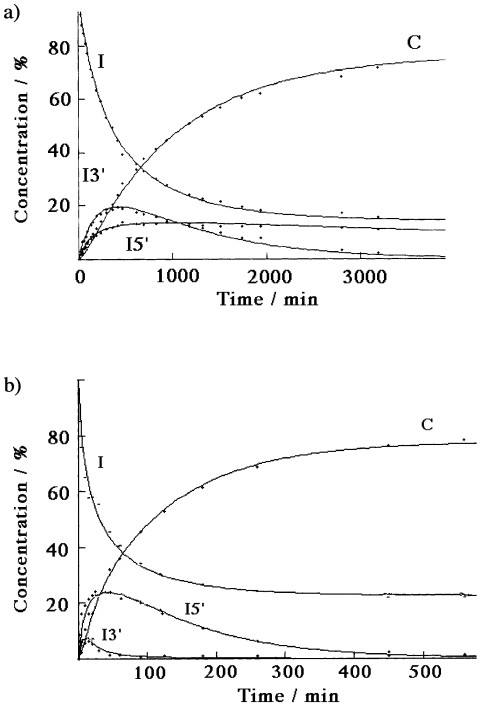


Figure 1. Typical experimental and calculated concentration curves for runs of the reaction systems (**I**+**2**) (a), (**I**+**3**/**4**) (b), in 0.1 M $NaClO_4$ at 293 K. Initial concentrations of oligonucleotide and platinum complex were 0.1 mM. For a) the pH was kept within the limits $pH\ 4.5 \pm 0.1$ by addition of $HClO_4$; for b) the initial pH of 5.6–6.8 was adjusted by addition of NaOH and subsequently left to evolve and monitored.

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Table 1. Optimized rate constants [$M^{-1}s^{-1}$] for platination of the hairpins **I** and **II** with the platinum complexes **2**–**5** at 293 K in 0.1M $NaClO_4$ (standard deviations in parentheses).

	TATGGTAT / ATACCATA T_4 (**I**)				TATAGTAT / ATATCATA T_4 (**II**)			
cis-$[Pt(NH_3)_2(H_2O)(Y)]^{2+/+}$	$k_{5'}$	$k_{3'}$	k_{tot}	$k_{5'}/k_{3'}$	$k_{5'}$	$k_{3'}$	k_{tot}	$k_{5'}/k_{3'}$
2 (Y = Cl^-)[a]	0.12(1)	0.28(1)	0.4	0.4	0.08(1)	0.19(2)	0.27	0.4
3 (Y = H_2O)[a]	18(2)	15(1)	33	1.2	1.5(3)	9(1)	10.5	0.2
4 (Y = OH^-)[b]	8.3(5)	6.3(4)	14.6	1.3	0.8(1)	4.0(2)	4.8	0.2
5 (Y = NH_3)[a]	1.7(2)	0.8(1)	2.5	2.1				

[a] pH = 4.5 ± 0.1. [b] Initial pH between 5.6 and 6.8. The pH evolution, monitored with time, was taken into account in the optimization procedure (see Experimental Section).

example) yielded the optimized rate constants shown in Table 1. The previously determined rate constants for the reactions of **3**,[18] as well as those related to the equilibrium $\mathbf{2} + H_2O \rightleftharpoons \mathbf{3} + Cl^-$ were fixed.[32] Reoptimization of these rate constants along with the other variables did not alter any values significantly. Chloride anation of **2** to **1** with the chloride liberated during the reaction could be neglected, since the value of the product of the anation rate constant (k_{-1} in Scheme 1, $\approx 4.5 \times 10^{-3}\,M^{-1}s^{-1}$) and the maximum chloride concentration (10^{-4}M) was negligible with respect to the rate constant for hydrolysis of **2** (k_2 in Scheme 1, $\approx 1.8 \times 10^{-5}\,s^{-1}$) at any point of the reaction.[32]

The platination rate constants specific for the aquahydroxo species **4** can be obtained from kinetic measurements at neutral pH, where **4** is preponderant over **3**. Combination of the results from such an experiment with those of an experiment at acidic pH enables one to determine the specific rate constants for **3** and **4**.[33] The experimental difficulty consists in maintaining a constant pH in the range between 6 and 7. Adjustments of pH by addition of diluted $HClO_4$ by hand or using a pH-stat did not work satisfactorily in our hands, since even very small additions caused significant pH jumps, or the acid had to be so diluted that the volume increase perturbed the measurements. Unlike other authors,[12, 13, 16, 24] we refrained from using phosphate or other buffers, since they contain potential ligands for platinum.[34] Specifically, the frequently used phosphate buffer has been clearly shown to perturb the kinetics of DNA platination reactions.[22, 35] In order to circumvent the problems of pH maintenance, we adjusted the pH at the beginning of the reaction and let it evolve without further intervention. The pH was measured at different time intervals, and a fit function was used to describe the pH–time curve. This function was then inserted into the calculation of the actual concentrations. From the H_3O^+ concentration thus determined, the distribution of the unreacted platinum complex between **3**, **4**, and *cis*-$[Pt(OH)_2(NH_3)_2]$ could be determined at any time point (the corresponding mathematical formulas are given in ref.[26]). Similarly, the ratios between aqua and hydroxo-monoadducts could be calculated from the H_3O^+ concentration and the corresponding acidity constants. The latter were constrained to be equal for the 5′ and 3′ monoadducts (the experimental data did not allow an independent refinement of both acidity constants) and were treated as a single variable. This variable was refined to values between 10^{-8} and 10^{-7}M; the estimated error bars were up to 80%, which obviously reflected the fact that at the experimental pH, the monoadducts were largely in the acidic aqua form and the exact K_a value had only a limited impact on the goodness-of-fit.

Platination reactions

A) Effect of complex charge: limited predictive value of molecular electrostatic potentials calculated at N7 lone-pair sites: Table 1 shows that all platination reactions are considerably (20–150 times) slower with **2** than with **3**. This could be, at first glance, because **2** is a monocation, whereas **3** is a dication. However, the difference of one order of magnitude observed between the rate constants for **3** and **5**, both dicationic, indicates that the charge of the platinum compound is not the sole, and probably not even the main factor determining the reactivity. This is further confirmed by the observation that deprotonation of **3** to **4** reduces the platination rate constants only by a factor of ≈ 2.

Pullman et al. used a combination of ab initio calculations and a multipole expansion procedure to determine molecular electrostatic potentials (MEP) around a central GC base-pair within a mini-B-DNA helix consisting of three base pairs.[36] They quantified the contribution to the MEP at the potential minima near the electronegative atoms. These relative contributions at the potential minimum corresponding to the central guanine N7 lone pair were $-6.1\,kcal\,mol^{-1}$ for an AGT sequence (complementary strand omitted for clarity), $-10.6\,kcal\,mol^{-1}$ for TGG, and $-12.5\,kcal\,mol^{-1}$ for GGT. The smaller negative contribution to the N7 potential of AGT compared with TGG and GGT is in agreement with the smaller reactivity that we found for the guanine of the AG sequence of **II** compared with that determined for both GG guanines of **I**. On the other hand, the MEP rating between TGG and GGT agrees only with the GG (**I**) reactivities towards **2** but not with those towards **3**, **4**, and **5**. The fact that replacement of the spectator ligand Y (Cl^- to OH^-, with the same charge) can reverse the order of reactivities clearly shows that electrostatic contribution is not the factor determining the kinetics of these reactions.

B) Effect of hydrogen bonding: The reactivity of the platinum complexes towards the GG and AG purines of **I** and **II** decreases in the order $\mathbf{3} > \mathbf{4} > \mathbf{5} >> \mathbf{2}$. This series parallels the ability of the different ligands (Table 1) to donate hydrogen bonds, that is high in the case of H_2O and OH^-, considerably less significant for NH_3,[37] and absent for Cl^-. Hydrogen

bonding between platinum ligands and the O6 atom of guanine would in fact yield a plausible rationale for the stabilization of the transition state (which is supposed to be pentacoordinate). In the case of adenine platination, such a hydrogen bond is not possible. Table 1 shows indeed that from **3** to **2**, the rate constants for adenine platination decrease considerably less than those for guanine platination, but the series **3** > **4** >> **2** still holds (we have no data on adenine platination by **5**). On the one hand this could be because of a charge effect (favoring **3**), and on the other hand because of hydrogen-bonding interactions between the platinum ligands and electronegative groups of the nucleotides adjacent to the adenine which is platinated.

C) Reactions with the chloroaqua species 2: direct substitution versus the solvolysis pathway: In acidic solution, **2** can react with an oligonucleotide either directly or through solvolysis to **3**. The rate constants listed in Table 1 prove that the solvolysis of **2** to **3** cannot be neglected. At the beginning of the reaction between **2** and **I**, for instance, **2** is consumed with a rate of $(k_{5'}+k_{3'})[\mathbf{I}][\mathbf{2}] = 0.40 \times 10^{-8}\,\text{M}\,\text{s}^{-1}$ through a direct reaction with **I** and with a rate of $k_{\text{Ptaq}}[\mathbf{2}] - k_{\text{Ptan}}[\mathbf{3}][\text{Cl}^-] = 0.17 \times 10^{-8}\,\text{M}\,\text{s}^{-1}$ by hydrolysis to **3**, since at $t = 0$, $[\mathbf{I}] = [\mathbf{2}] = 10^{-4}\,\text{M}$ and $[\text{Cl}^-] = 0$. Thus, 30% of **2** is hydrolyzed to **3**. In the course of the reaction, [**I**] and [**2**] decrease while $[\text{Cl}^-]$ increases; the balance between the two pathways then depends on the instantaneous concentration of **3**. Under our reaction conditions, the fraction of **2** hydrolyzed increased continuously, and the total amount of **I** that reacted with **3** was ≈40%. Thus, neglecting the solvolysis pathway, as practiced in previous studies, yields erroneous rate constants.

D) GG versus AG platination: A comparison of the platination rate constants (Table 1) shows that the differentiation between GG and AG depends on the platinum ligands. Whereas **3** and **4** react with **I** approximately three times faster than with **II** (compare the sums $k_{5'}+k_{3'}$), **2** reacts with both oligonucleotides at comparable rates. This indicates that if **2** interacted with DNA, one should find comparable amounts of GG and AG adducts. Moreover, since **2** reacts only twice as slowly with the adenine as with the guanine of AG, and since the hydrolysis of the adenine-bound monoadduct is relatively slow (vide infra), one should find a substantial number of monoadducts bound to adenine. Neither expectation is in agreement with experimental observations, and casts doubt on the hypothesis which assumes **2** is the actual species that binds to DNA.

E) The two GpG guanines of the TGGT sequence of I react with similar rates: From a comparison of the specific rate constants determined for the platination of the individual guanines of **I** ($k_{5'}$ versus $k_{3'}$), we observe that all three complexes **3**–**5** react with both guanines of **I** with comparable rates and differ at most by a factor of 2, that is, none of them shows a pronounced preference for the platination of the 5′-G or the 3′-G. This is in contrast with the results obtained previously for the self-complementary duplex $d(TTGGCCAA)_2$,[15] where **3** showed a 12-fold preference for the 5′-G. This difference could either reflect the influence of the bases flanking the GG sequence, or originate in a particular structure of the palindromic sequence, as suggested by an NMR investigation. A kinetic study of **3** with the sequence TGGC embedded in a hairpin-duplex analogous to **I** has revealed a similar (≈10 fold) 5′ over 3′ selectivity (V. Bas, J. Kozelka, J.-C. Chottard, ongoing work). The most significant effect of exchanging the 3′-thymine of TGGT with a cytosine (yielding TGGC) is an approximate fivefold rate decrease of the 3′-platination. This may indicate that the 3′-cytosine destabilizes the transition state for the 3′-platination. We are currently using molecular modeling in a search for platinum ligand–groove interactions which could effect such a destabilization.

Hydrolysis of chloro-monoadducts

For both **I** and **II**, we observe that the hydrolysis of the chloro-monoadduct bound to the 3′-base proceeds considerably faster than that of the 5′-monoadduct (Table 2). In order to appreciate the impact of the sequence environment, it is useful to compare these rate constants with those determined at the same temperature and ionic strength for the aquation of

Table 2. Optimized rate constants for the aquation and chelation of the chloromonoadducts formed between the hairpins **I** and **II** and the platinum complex **2** at 293 K in 0.1M $NaClO_4$ (standard deviation in parentheses).

	Aquation[$10^{-5}\,\text{s}^{-1}$]			Chelation [$10^{-3}\,\text{s}^{-1}$][a]		
	$k_{5'a}$	$k_{3'a}$	$k_{5'a}/k_{3'a}$	$k_{5'c}$	$k_{3'c}$	$k_{5'c}/k_{3'c}$
TATGGTAT / ATACCATA T_4 **I**	0.19(1)	1.7(1)	9	0.18(1)	0.9(2)	10.5
TATAGTAT / ATATCATA T_4 **II**	0.26(6)	0.65(4)	2.5	0.3(3)	0.08(1)	0.3

[a] Ref. [18].

cis-$[PtCl(NH_3)_2(dGuo)]^+$ ($1.4 \times 10^{-5}\,\text{s}^{-1}$) and of *cis*-$[PtCl(NH_3)_2(5'\text{-Me-dGMP})]$ ($0.9 \times 10^{-5}\,\text{s}^{-1}$).[26] This comparison reveals that whereas the hydrolysis of the 3′-chloro-monoadducts proceeds with rates in the expected range, that of the 5′-chloro-monoadducts is slowed down.

This result can be related to the previous finding made by Leng's group that monoadducts with a *cis*-$PtCl(NH_3)_2^+$ residue bound to the G* guanine of sequences d(XG*C)-(GCY) within duplex DNA rearrange to interstrand di-adducts with rates dependent on the nature of the XY base-pair.[38] Since chloride arrests this rearrangement, one can conclude that the rate-determining step is the hydrolysis of the chloro-monoadduct, and that the nature of the XY base-pair modulates the hydrolysis rate. The monoadduct where X was a thymine hydrolyzed with the slowest rate. Another indication that the hydrolysis of chloro-monoadducts is sequence-dependent emerged from the work of Berners-Price et al., who investigated the reaction between *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**2**) and the duplex d(ATACAT*GG*TA-CATA)-d(TATGTACCATGTAT) (the platinated guanines are printed in italics) by means of [^{1}H,^{15}N] NMR. They

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observed that the peaks due to the chloro-monoadducts decayed with the rate constants of $0.7 \times 10^{-5}\,s^{-1}$ (5′) and $4.6 \times 10^{-5}\,s^{-1}$ (3′) at 25 °C.[16, 17] These rate constants mainly reflect the hydrolysis of the chloro ligands, since the chelation of the corresponding aqua-monoadducts was observed to be one order of magnitude faster.[17] In all the above cases of sequence-dependent hydrolysis of chloro-monoadducts, the more (or most) slowly hydrolyzing adduct had a thymine on the 5′-side. Our results show that duplex DNA may slow-down, in a sequence-dependent manner, the hydrolysis of platinum chloro-monoadducts. More data are needed to obtain further insight into this effect which can reach one order of magnitude.

Chelation reactions

The chelation rate constants were measured with the restrained system, which involve the reactions between **3** and **I** or **II**.[18] As we have pointed out,[18] in all the investigated duplex oligonucleotides platinated on a GG-sequence, the 3′-monoadduct was chelated ≈10 times faster than the 5′-monoadduct. Possible causes for this difference have been discussed previously.[15] The chelation of the 3′-monoadduct of **II** is about 10 times slower than that of the 3′-monoadduct of **I**, in accordance with the lower reactivity of adenine with respect to guanine.

Comparison with other studies

Berners-Price, Sadler et al. have used [^{1}H,^{15}N] NMR spectroscopy to study the interaction between the different cisplatin forms and double-stranded oligonucleotides in aqueous solution.[16, 17, 24] Comparisons between their rate constants and ours are not straightforward, i) since they used simplified kinetic models, and ii) because of differences in temperature, ionic strength, and pH, and of the presence of phosphate buffer in some experiments. For the reaction between **2** and the duplex d(ATACAT*GG*TACATA)-d(TATGTACCATGTAT) in 9 mM sodium phosphate at pH 6 and 298 K, for instance, the platination rate constants $k_{5'} = 0.20 \pm 0.01\,M^{-1}s^{-1}$ and $k_{3'} = 0.54 \pm 0.02\,M^{-1}s^{-1}$ were found.[16, 17] Whereas a comment on the absolute values is difficult to make (since they depend on experimental conditions), the ratio $k_{3'}/k_{5'}$ of 2.7 is in good agreement with the ratio of 2.3 that we have found for the analogous reaction with **I** (Table 1). It has to be noted, however, that the rate constants measured for d(ATACAT*GG*TACATA)-d(TATGTACCATGTAT) contain a contribution owing to the pathway of hydrolysis of **2** to **3/4** (Scheme 2), which was not taken into account explicitly.

In a recent study, Davies et al. investigated the reactions of the palindromic duplexes d(AATT*AG*TACTAATT)$_2$ and d(AATT*GA*TATCAATT)$_2$ with **1** and **3**, respectively.[24] For the reaction between d(AATT*AG*TACTAATT)$_2$ and **3**, they found a surprisingly low guanine platination rate constant ($0.419 \pm 0.009\,M^{-1}s^{-1}$ in 0.1 M $NaClO_4$ at 288 K and pH 4.9), whereas for the platination with **2** (formed through hydrolysis of **1**), an unusually high rate constant was determined ($1.06 \pm 0.06\,M^{-1}s^{-1}$ in 9 mM sodium phosphate at 298 K and pH 6). Even if one takes the different reaction conditions into account (the higher temperature and lower ionic strength accelerating, the higher pH and the phosphate ions slowing down platination with **2**), the higher rate constant found for **2** contradicts common experience indicating that **3** reacts with DNA guanines one to two orders of magnitude faster than **2**.[22, 23] Another surprising observation is that during the reactions of d(AATT*AG*TACTAATT)$_2$ and d(AATT*GA*TATCAATT)$_2$ with cisplatin, the concentration of the latter decayed with considerably different initial slopes (Figure 2 of ref.[24]), whereas this slope should reflect uniquely the rate of cisplatin hydrolysis and thus be identical in the two reactions. We therefore believe that the rate constants issued from this work need clarification.

Conclusion

The aim of this work was to examine how platinum ligands of the hydrolyzed derivatives 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**), control the kinetics and selectivity of DNA platination. These three complexes of the general formula *cis*-[$Pt(NH_3)_2(H_2O)(Y)$]$^{2+/+}$ ($Y = Cl^-$, H_2O, OH^-), as well as the triammine derivative [$Pt(NH_3)_3(H_2O)$]$^{2+}$ (**5**; $Y = NH_3$), were made to react with two oligonucleotides, **I** and **II**, bearing the two platinum binding sites, GG and AG, respectively, in an identical environment. This is the first determination of site-specific platination rate constants for **2**–**5**, carried out under the same conditions.

The first major result emerging from this comparative study is that whereas the aqua/hydroxo derivatives **3** and **4** show a clear 3:1 GG over AG binding preference, only a weak selectivity is observed with the chloro complex **2**. Since it has been established in numerous experiments that the reaction between cisplatin and DNA yields three times more GG than AG adducts, this result contradicts the common view[39–41] that **2** is the species actually reacting with DNA bases. This would suggest that cisplatin has to undergo double hydrolysis before reacting with DNA. On the other hand, work from several groups did provide results interpretable as evidence for direct interaction between **2** and DNA. For instance, Johnson et al. determined apparent first-order rate constants for the reaction between **2** and calf thymus DNA that were linearly dependent on DNA concentration.[22] In other DNA platination experiments, Schaller et al.[23] and Bancroft et al.[21] found that in the presence of excess DNA, the concentration of **2** decayed faster than the corresponding uncatalyzed hydrolysis to **3**. A question that has not yet been addressed is whether the hydrolysis of **2**, when **2** is associated by electrostatic forces with the DNA surface, proceeds with the same rate as in monovalent electrolytes in which kinetic measurements are mostly carried out. It has been shown that the large negative potential of DNA repels anions and concentrates cations,[42] including H_3O^+,[43–45] and also depletes Cl^- from the DNA surface and causes it to be more acidic than the bulk solution. Such conditions push the aquation equilibria towards the hydrolyzed forms, and the protolytic equilibria towards the aqua species (i.e., favoring **3** over **2** and **4**). In addition, the polar DNA residues and the negative charge residing on the phosphate groups may accelerate substitution reactions by

means of electrostatic catalysis. This hypothesis remains to be substantiated by more experimental data.

The second major result is that the platination rate constants seem to be more sensitive to the hydrogen bonding potential of the platinum complex than to its charge. The order of platination rates within the series *cis*-$[Pt(NH_3)_2(H_2O)(Y)]^{2+/+}$ is **3** > **4** > **5** >> **2** (Y = H_2O > OH^- > NH_3 >> Cl^-), which is the order of decreasing hydrogen-bond donor ability. This suggests that hydrogen-bonding interactions play a major role in the transition state of the first GN7 coordination. It is noteworthy that the exchange of the sole Y ligand modifies the platination rate constant for a given DNA base by a factor up to 150. On the other hand, it is interesting to note that complexes **3** and **4**, whose Y ligand is H_2O and OH^-, respectively, differ only by a factor of two for their platination rates and exhibit identical selectivities between the purines of **I** and **II**.

According to the current model for cisplatin–DNA interaction in vivo, cisplatin passes through the cell membrane in its dichloro form **1**. Inside the cell, the hydrolysis to **2** occurs, favored by the low chloride concentration in the cytoplasm; this step has been shown by numerous authors to be rate-determining for DNA platination. Recently, Jennerwein and Andrews have demonstrated for 2008 human ovarian carcinoma cells that replacement of 92% of the intracellular chloride by nitrate does not affect the rate of platinum binding to DNA.[46] This result suggests that chloride anation of **2** back to **1** is negligible, that is, the equilibrium between **1** and **2** has no time to establish. This would be expected anyway since the hydrolysis of **1** is very slow ($\tau_{1/2}$ = 1.9 h at 37 °C). Once hydrolyzed to **2**, the monocationic platinum complex will probably rapidly associate with the polyanionic DNA in the nucleus, forming an outer sphere complex. Our data are in line with a kinetic scheme in which the second hydrolysis takes place within this outer sphere complex, before the first coordination of a nucleobase has occurred. In the acidic conditions prevailing in the DNA grooves,[43–45] **3** would remain largely in the diaqua form, and the site-selectivity of nucleobase binding would thus be dictated by interactions between its ligands and the DNA residues in the major groove.

Certainly, more work is needed to make sure that the 3:1 GG over AG preference observed for **3** and **4**, and the lack of clear preference recorded for **2**, reflect general sequence selectivities of the hydrolyzed cisplatin forms. Another point that has to be addressed in future work is the rate of hydrolysis of **2** associated with double-stranded DNA.

Experimental Section

Starting materials: The oligonucleotides were synthesized as ammonium salts; their purity was checked by capillary electrophoresis and analytical HPLC by the group of T. Huynh Dinh (Institut Pasteur, Paris). Approximate concentrations were evaluated photometrically by using an average molar absorption coefficient ε_{260nm} of 8000 $M^{-1}cm^{-1}$ per base. Cisplatin was kindly provided by Johnson-Matthey. Solutions of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$[14, 15] and *cis*-$[PtCl(NH_3)_2(H_2O)]^+$[30] were prepared as previously described. Perchloric acid and all salts were purchased from Merck.

Kinetic analyses: i) General procedure: The reactions were performed in $NaClO_4$ (0.1 M) at 20 °C, the initial concentrations were 0.12–0.15 mM for the oligonucleotides and 0.08–0.10 mM for the platinum complexes (a slight oligonucleotide excess was used in order to avoid bis-platination). At this low concentration, formation of hydroxo-bridged species is expected to be negligible even at pH close to neutral.[33] Aliquots of the reaction mixtures were collected at several time points, quenched by addition of saturated KCl or KBr solutions, respectively, for reactions with **II** and **I**, and stored at liquid-nitrogen temperature until they were analyzed by reversed-phase HPLC.[11] These quenching conditions have been shown to trap all the aqua intermediates and to avoid evolution of the reaction.[11, 18, 47] The reactions with **2**, **3**, and **5** were carried out at pH of 4.5 ± 0.1, adjusted by the addition of small amounts of diluted $HClO_4$. For the reactions with **4**, the pH of the oligonucleotide was adjusted between 5.6 and 6.8 with diluted NaOH; then the solution of **3** was added and the pH of the reaction mixture measured. The pH–time curves were determined by measuring the pH at different time intervals. The final pH lie between 6.7 and 7.3. The pH electrode (Metrohm 691, filled with 0.1 M $LiClO_4$ as internal electrolyte) was *not* left permanently immersed in the reaction vessel, in order to avoid diffusion of the electrolyte into the vessel. A typical pH–time curve for one reaction is presented in Figure 2.

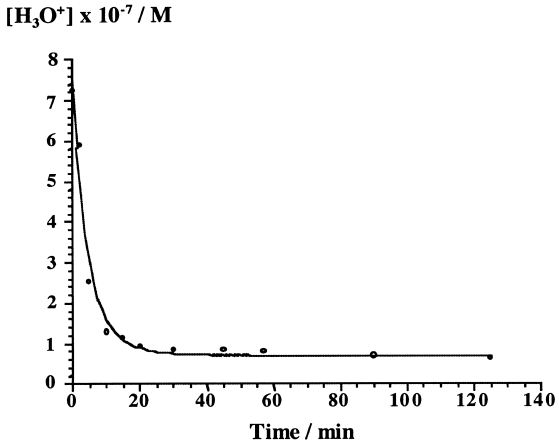

Figure 2. pH–time curve recorded for the experiment (**II** + **3/4**) as a function of time. (●) experimental measurements; (——) fit function $[H_3O^+] = a + b/(\text{time}+c)^d$, with the coefficients *a*, *b*, *c*, and *d* optimized using the program Kaleidagraph.

ii) Reversed-phase HPLC analysis of the reaction mixtures and identification of the products: HPLC analysis of the reaction aliquots was performed with a Beckman 126 pump coupled to a Beckman diode array detector 168 and a System Gold V810 integrator. The system was connected to a Rheodyne 7725i valve. A POROS R2/H (100 × 4.6 mm i.d., 10 μm) column (PerSeptive Biosystems GmbH) was employed for the analysis of the adducts from **II**, and a stainless steel column Nucleosil C8 (150 × 4.6 mm i.d., 5 μm, 300 Å, Colochrom, France) for the separation of the adducts from **I**. Operating conditions are given in the caption of Figure 3 and in that of Figure 3 of ref.[18]. Relative concentrations were determined from the ratios of the peak areas. The detection wavelength of 245 nm was chosen close to the quasi-isosbestic points observed for the reactions involving **2** and **3/4**. For the reaction between **I** and **5**, which did not alter the absorption spectrum to a detectable extent, the detection wavelength was that of the absorption maximum (260 nm).The identification of the intermediate monoadducts and the final NG*cis*-Pt chelate was achieved by enzymatic digestion using the VPD 3′-exonuclease which stops at a platinated nucleoside leaving a 3′-OH free end.[14, 48] The monoadducts and chelate formed upon reaction and quenched at the time of their maximum concentrations in the reaction mixture, were collected at the outlet of the column and digested by 20 mL of VPD without addition of buffer at 37 °C. After lyophilization, the digested fragments were identified by mass spectroscopy using the MALDI ionization method (Figure 4). The MALDI spectra have been performed at University Pierre et Marie Curie on an in-house built MALDI spectrometer with a linear time of flight (TOF) analyzer as described previously.[49]

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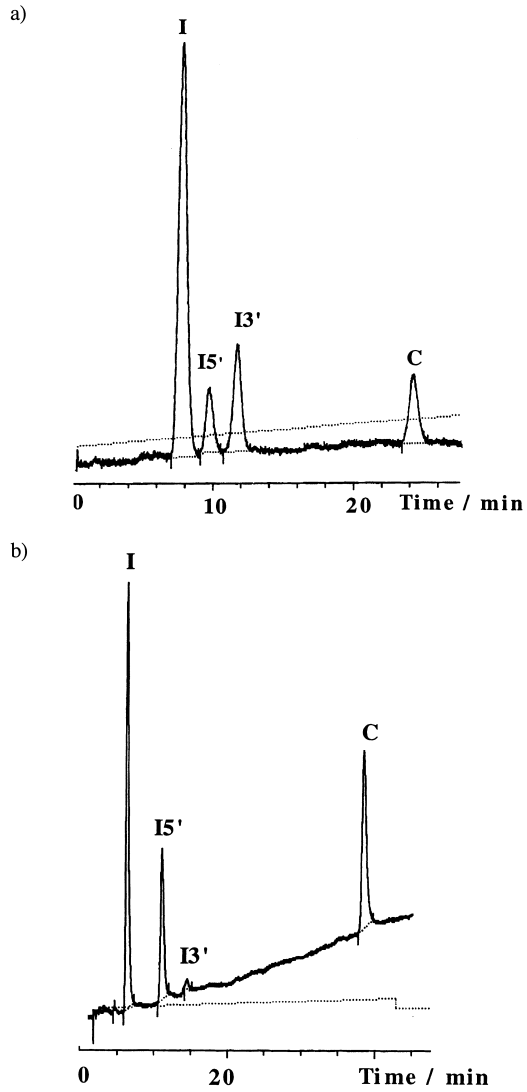

Figure 3. Typical chromatograms of platination mixtures quenched by halide ions. Reaction conditions: 0.1M $NaClO_4$; pH4.5 ± 0.1 T = 273 K. a) Reaction between **I** and **2** quenched at t = 135 min by excess KBr. POROS R2/H column. Mobile phase, solvent A: NH_4Br 1M, urea 1M, triethylammonium acetate buffer 10^{-2}M, pH 7, solvent B: CH_3CN/H_2O (50/50). Solvent B gradient: 7–20% from 0 to 50 min; flow rate 3 mL min^{-1}; column temperature 40 °C; detection wavelength: 245 nm. b) Reaction between **I** and **3/4** quenched at t = 30 min by excess of KBr. Nucleosil C8 300 Å (150 × 4.6 mm, 5 μm) column. Mobile phase, solvent A: NH_4Br 0.5M, ammonium acetate buffer 0.04M, pH 5.0; solvent B: CH_3CN. Solvent B gradient: 7.5% for 3 min, 7.5–10% from 3 to 43 min; flow rate 1 mL min^{-1}; column temperature 50 °C; detection wavelength: 260 nm. I5′: 5′-monoadduct, I3′: 3′-monoadduct, C: chelate.

iii) Data analysis: The rate constants were calculated by numerical integration of the differential equations using the program ITERAT.[50] The reaction schemes for the platination of **I** and **II** with **2** and with **4** are shown in Schemes 2 and 3. Both analyses started with the evaluation of the corresponding reaction with **3**, described previously.[18] The mathematical adaptations necessary to introduce the time-dependence of the H_3O^+-concentration have been described by Weber et al.[26]

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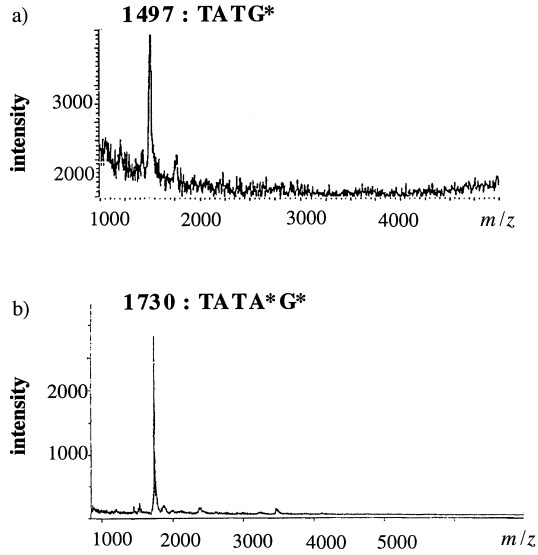

Figure 4. MALDI mass spectra: a) intermediate monoadducts (I5′G) of **I** and b) final AG*cis*-Pt chelate of **II**. (Matrix: THAP, 2,4,6-trihydroxyacetophenone).

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