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Kinetic aspects of interactions between DNA and platinum complexes

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Abstract

Kinetic studies on reactions between oligonucleotides and platinum complexes related to the antitumor drug cisplatin are described. These studies were motivated by the sequence-selectivity observed for the reaction between cisplatin and DNA. Comparison of oligonucleotide platination rate constants measured for the three complexes *cis*-$[PtCl(NH_3)_2(H_2O)]^+$, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, and $[Pt(NH_3)_3(H_2O)]^{2+}$ suggests that the hydrogen bond donating capacity of the platinum ligands enhances the platination rate. The binding preferences of *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ indicate that the latter species is unlikely to be the major species interacting with DNA in vivo. This conjecture is corroborated by reactivity and availability considerations. We also address the sequence-dependence observed for the hydrolysis of chloro-monoadducts and for the conversion of aqua-monoadducts to diadducts, and discuss possible mechanisms.

Keywords: Platinum complexes; DNA; Kinetics; Antitumor drugs

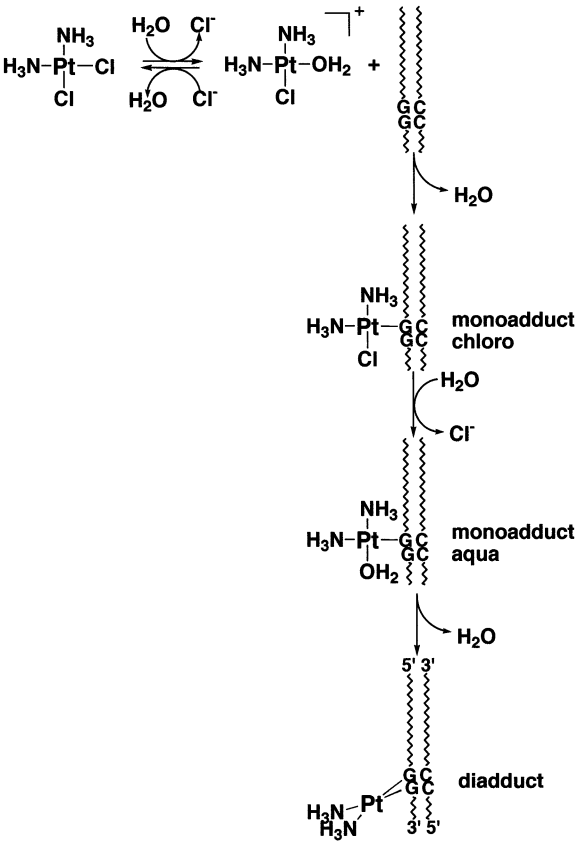
1. Introduction

Cisplatin, *cis*-$[PtCl_2(NH_3)_2]$, is one of the most widely used antitumor drugs [1]. It is a square-planar Pt(II) complex featuring two inert Pt–N bonds and two relatively labile Pt–Cl bonds. The electrophilic character of the central ion allows *cis*-$[PtCl_2(NH_3)_2]$, after entering the cell, to react with nucleophilic groups of the cellular components. A number of observations have indicated that the antitumor activity of cisplatin is related to its ability to bind to DNA [2,3].

The binding of cisplatin to DNA consists of a sequential replacement of the chloride ligands by the N7 atoms of guanine or adenine bases. Adenine coordination has been observed only in the second step. It has been established that purine bases do not replace chloride from Pt(II) directly, but predominantly via a solvent-assisted mechanism [4]. Thus, the kinetics of the first step has been shown by in vitro experiments to be of first order, with a rate constant corresponding to the hydrolysis of *cis*-$[PtCl_2(NH_3)_2]$ to *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ [5–7]. Similarly, in studies where the decay of the chloro-monoadducts could be observed [7–9], the

rate constant (of the order of 10^{-5} s^{-1} at 37°C) was as expected for the hydrolysis of a monocationic Pt(II)–chlorotriamine species.

The generally assumed mechanism for cisplatin–DNA interactions is schematically drawn in Scheme 1. Both the formation of platinum monoadducts, and the monoadduct-to-diadduct conversion show sequence-dependent kinetics. The first platinum-N7 bond is formed preferentially on guanines involved in G_n tracts ($n \geq 2$). The second preferred binding sites appear to be guanines 3′ to an adenine. Since the platinum–nitrogen bond is generally inert, this selectivity of the initial binding step determines the final distribution of the diadducts. Thus, after platination of DNA in vitro [10–12], in cell cultures [13], or in the white blood cells of cancer patients [14–17], the main adducts are GG (60–65%) and 5′-AG-3′ (ca. 20%) intrastrand chelates. A sequence-dependent kinetics was also observed in experiments where isolated monoadducts bearing a *cis*-$PtCl(NH_3)_2^+$ moiety bound to a guanine were allowed to rearrange to diadducts [18].



Scheme 1.

The present contribution focuses on oligonucleotide studies that were conducted in order to evaluate the rules governing the sequence-dependent phenomena mentioned above and to obtain indications about the possible mechanisms. The article is structured as follows. After a brief summary of methods that have been used in kinetic studies on platinum–DNA interactions (Section 2), Section 3 presents results related to sequence-selective binding of cisplatin to DNA, with emphasis on our own work presented at the ICCC-33 conference. This section includes kinetic studies of the first platination step (Section 3.1), investigations relevant to the sequence-dependence of the monoadduct-to-diadduct conversion (Sections 3.2 and 3.3), and possible mechanistic implications of these studies for the DNA platination in vivo (Section 3.4). A brief conclusion terminates the article.

2. Methods used to monitor Pt–DNA interactions

A variety of physical methods have been used to monitor the reactions between platinum complexes and nucleic acids or their constituents. We try to list them here briefly, without the desire for completeness.

2.1. Chemical trapping agents

When non-in situ analytical techniques are employed, it is necessary to arrest the reaction in aliquots that are withdrawn from the reaction mixture at various time intervals. This can be done (i) by adding a quenching agent inactivating the reactive species; (ii) by freezing the samples; (iii) by precipitation, or by a combination of the three methods. Removal of unreacted platinum complex by centrifuging the DNA through a Sephadex column was also used [11]. A variety of ligands blocking the reactive sites on platinum, including halogenide ions [19–21], hydroxide [22], cyanide [23], thiocyanate [24], guanosine [25], thiourea [26,27], and ammonium bicarbonate [27] have been employed. Some of these agents are problematic, as has been discussed by Lepre and Lippard [28].

2.2. Markers

2.2.1. Radioactive labels

Radioactive isotopes provide very sensitive probes allowing for the detection of picogram quantities. Various radiolabeled compounds have been used in kinetic studies of Pt–DNA interactions. These include:

1. platinum complexes labeled with ^{195m}Pt [29]
2. ^{3}H-labeled nucleoside triphosphates [22,23] or [$PtCl_2$(en)] [11]
3. ^{14}C-labeled cyanide [23] or guanosine [25]
4. [γ-^{32}P]ATP or [5′-^{32}P]pCp used for classical DNA-labeling.

The half-life times of these radioisotopes are: ^{195m}Pt, 4.1 days; ^{3}H, 12.26 years; ^{14}C, 5730 years; ^{32}P, 14.3 days [30].

2.2.2. Intercalating dyes

The fluorescence enhancement of DNA-intercalated ethidium is quenched upon formation of bifunctional platinum complexes with DNA [31,32]. A linear relationship between the percentage of fluorescence decrease and the r_b factor has been observed for both *cis*- and *trans*-$Pt(NH_3)_2^{2+}$-binding to DNA up to $r_b = 0.20$ [32–35]. Measuring the fluorescence of the ethidium–DNA complex thus allows the Pt–DNA monoadduct-to-diadduct conversion to be monitored [23,33,35,36].

2.3. Analytical methods

2.3.1. Optical spectroscopy

2.3.1.1. UV spectrophotometry. UV spectrophotometry is well-suited for the monitoring of reactions accompanied by the modification of a chromophore. For instance, replacement of a chloro ligand of platinum by H_2O results in a ca. 30 nm blue-shift of the d–d bands [37]. Thus, the hydrolysis of platinum chloro complexes can be followed at wavelengths corresponding to the ligand-field spectrum of $PtCl_nN_2O_{2-n}$ chromophores ($n = 0$–2), i.e. between 240 and 400 nm. This possibility has been explored in determinations of the hydrolysis rate constants of cisplatin [38–40] and related complexes ([41,42] and Refs. therein). In complexes with (oligo)nucleotides, the platinum ligand-field region is obscured by the nucleobase absorption bands. Nevertheless, the reactions of $[Pt(dien)(H_2O)]^{2+}$ with 5′-GMP and with d(GpG) could be followed by monitoring the absorbance at 294 nm [43], and at 280 and 254 nm [44], respectively.

When a platinum complex reacts with DNA, not only the transitions within the platinum chromophore but also those involving the DNA bases are modified. This has been exploited in kinetic measurements by several authors [5,45]. Specifically, it has been observed that the reaction between *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and DNA leads to an increase of absorbance at 272 nm [45].

2.3.1.2. Circular dichroism spectroscopy. Circular dichroism spectra of double-stranded DNA are modified upon the binding of platinum complexes according to the binding mode [33,46–49]. For instance, the positive ellipticity at 280 nm decreases monotonically with time when calf thymus DNA is reacted with [PtCl-(dien)]Cl or *trans*-$[PtCl_2(NH_3)_2]$ at an input ratio r_o of 0.08 [46]. In contrast, upon binding of *cis*-$[PtCl_2(NH_3)_2]$ at the same r_o, the amplitude of the 280 nm band decreases in the initial phase (attributable to monofunctional binding), reaches a maximum within 5–10 min, and subsequently increases [46]. These effects are summarized in figure 5 of [47]. The marked change in ellipticity around 280 nm upon the reaction between DNA and platinum complexes has been used to follow the kinetics of these reactions [23,46,47].

2.3.1.3. Fluorescence spectroscopy. As outlined in Section 2.2.2, measurement of the fluorescence of DNA-intercalated ethidium has been used to follow the Pt–DNA monoadduct-to-diadduct conversion [23,33,36,50].

2.3.2. Nuclear magnetic resonance

Integration of NMR signals can be used for concentration measurements and hence for kinetic studies, provided that (i) the peaks due to reactants, intermediates (if any), and final products can be sufficiently resolved and (ii) the time necessary for acquisition is short with respect to the reaction rate. NMR combines the advantages of a non-invasive method with the possibility of measuring the concentrations of several species at the same time. On the other hand, continuous adjustments during the reaction (e.g. of pH) are virtually impossible with current spectrometers.

^{1}H-NMR spectroscopy has been used in reactions between platinum complexes and nucleotides, the ribose H1′ [43] or guanine H8 proton signals [51] serving as probes.

Kerrison and Sadler monitored the solvolysis of cisplatin in DMSO by means of ^{195}Pt-NMR [52]. Bancroft et al. employed cisplatin enriched in ^{195}Pt in a DNA platination study [7]. Using very fast scans (200 000 transients h^{-1}) together with a 90° pulse and a 100 kHz spectral window, and employing relatively concentrated solutions (32 mM in Pt and 455 mM in DNA nucleotides), they were able to quantify the platinum-containing reactants, intermediates, and final products. ^{195}Pt-NMR spectroscopy profits from the large chemical shifts accompanying ligand substitution reactions on Pt(II) complexes. On the other hand, as a consequence of the non-uniform excitation over the large spectral window, and of the fast scanning (which does not allow the Pt nuclei to relax completely), intensities of different peaks cannot be used for calculation of relative concentrations in a straightforward manner [7].

Recently, Sadler's group applied two-dimensional [^{1}H,^{15}N]-HSQC-NMR spectroscopy to kinetic measurements on hydrolysis reactions [53] as well as on oligonucleotide interactions with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and *cis*-$[PtCl_2(NH_3)_2]$ [54–56]. The results were checked using a parallel HPLC study in one case [56]. Both methods agreed in the order of magnitude of the determined rate constants, but the NMR values were throughout higher than those measured by HPLC. As a possible source for this discrepancy, the paucity of the NMR data at the beginning of the reactions (caused by the relatively long acquisition times) was named [56].

2.3.3. Atomic absorption spectroscopy

Atomic absorption spectroscopy is a method commonly used for the quantitation of platinum bound to DNA. Typical operating conditions can be found in Refs. [23,57,58].

2.3.4. Mass spectrometry

ESI [19] and MALDI-TOF [20] mass spectrometric methods have been used as complementary tool for the identification of products occurring during the platination of oligonucleotides. Recently, Gonnet et al. pioneered in using ESI and MALDI-TOF mass spectrometry, together with enzymatic degradation, to monitor

the reaction between the single-stranded oligonucleotide d(TTGGCCAA) and $[Pt(NH_3)_3(H_2O)]^{2+}$ [59].

2.3.5. Pulse polarography

The Brno group has established that differential pulse polarography can serve to quantify the molar ratio of bound platinum per DNA nucleotide (r_b) [60,61]. Monitoring the polarographically determined r_b value was used to follow the kinetics of platination of calf thymus DNA with $[PtCl(dien)]^+$, *cis*-$[PtCl_2(NH_3)_2]$, and *trans*-$[PtCl_2(NH_3)_2]$ [46].

2.3.6. Chromatography

The formation of various platinum–DNA adducts as a function of incubation time can be followed using a quenching agent to arrest the reaction (Section 2.1), a nuclease to digest the DNA (Section 2.3.8), and either FPLC [12,14,62] or HPLC [12,22,24,26,45,58] to separate and quantify the digests. HPLC has also been used in studies of platinum complexes with nucleobases, nucleosides, and nucleotides ([63,64] and Refs. therein), as well as in those with oligonucleotides [19,21,65–68]. The power of HPLC to separate isomers of very similar structures, such as the two platinum monoadducts bound to either guanine of a GG sequence within an oligonucleotide, allows the individual rate constants for the formation of such monoadducts to be determined simultaneously with the evaluation of the rate constants for the two subsequent chelation reactions [19,21,56,66,69].

2.3.7. Electrophoresis

2.3.7.1. Gel electrophoresis. Polyacrylamide gel electrophoresis of radiolabeled DNA has been extensively used by Leng's group to follow the kinetics of rearrangement reactions of different platinum–DNA adducts [18,70,71]. Using denaturing conditions, intrastrand and interstrand platinum–DNA crosslinks can be readily distinguished.

2.3.7.2. Capillary electrophoresis. The quantification of adducts originating from the platination of a double-stranded DNA 18-mer by $[Pt(NH_3)_3(H_2O)]^{2+}$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ using capillary electrophoresis has been recently reported by Troujman and Chottard [68]. The authors employed capillary gel electrophoresis and capillary electrophoresis coupled with the micellar electrokinetic capillary chromatography (MECC) technique to determine the platination and (for *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$) the chelation rate constants. For the identification of the reaction products, they recurred to the classical method using HPLC and enzymatic digestion.

2.3.8. Enzymological assays

2.3.8.1. Enzymatic digestion. Nucleases such as nuclease P1 [11,12,14,58], Benzonase™ [58], DNase 1 [12,14], venom phosphodiesterase (VPD) [59,66,72,73], the

5′-exonuclease from calf spleen (SPD) [59], or alkaline phosphatase [58] were used to digest platinated DNA or oligonucleotides. The digested fragments were subsequently identified and quantified using chromatography (Section 2.3.6), mass spectrometry (Section 2.3.4) or gel electrophoresis (Section 2.3.7.1).

2.3.8.2. Replication mapping. The arrest of DNA synthesis by platinium mono- and diadducts has been exploited by Holler et al. in order to follow the platination kinetics [23,58]. Using a mixture of dATP, dCTP, dGTP, and [^{3}H]dTTP, these authors quantified the incorporation of tritium into DNA.

2.3.9. Immunochemical quantification of adducts

Adduct-specific antibodies allow the detection of the individual Pt–DNA adducts on the femtomol level. Fichtinger-Schepman and colleagues have adapted the competitive enzyme-linked immunosorbent assay (competitive ELISA) [13,14,62,74,75] and the immunoperoxidase staining procedure [75] for the time-dependent quantification of the different adducts in living cells.

2.3.10. Platinum binding to DNA detected by thickness–shear-mode acoustic wave sensor

Thompson et al. have attached double-stranded calf thymus DNA to the electrodes of a thickness–shear-mode acoustic wave sensor and measured the series resonant frequency shift after injection of cisplatin or transplatin solutions into the measurement cell [76]. The series resonant frequency shift versus time curves were analyzed in terms of first order processes and the rate constants compared to those obtained by Bancroft et al. using ^{195}Pt-NMR [7]. This comparison is not straightforward: whereas the acoustic wave sensor method is DNA-centered, and the observed pseudo-first-order rate constant therefore corresponds to the second-order rate constant multiplied by the concentration of the reacting platinum complex, the ^{195}Pt-NMR-based method yields a pseudo-first-order rate constant which is a product of the second-order rate constant and the concentration of DNA (see a comment by Jestin et al. on the fulfillment of pseudo-first-order conditions in the latter experiment [77]).

3. Results

3.1. Sequence-dependent binding of the hydrolyzed cisplatin forms to DNA

The identification of intrastrand GG (60–65%) and AG (ca. 20%) crosslinks as the major final adducts arising from cisplatin treatment of DNA in vitro [10–12] or in vivo [13,14] suggested that guanines involved in these sequences are more reactive than other guanines (initial attack of adenine bases of DNA on platinum is a rare

event [78]). A rate constant for the DNA platination could not be determined in these studies, since the rate-limiting step in the DNA–cisplatin interaction is cisplatin hydrolysis which precedes the second-order reaction [5]. Approximate second-order rate constants were inferred from in vitro experiments in which DNA was reacted with the monoaquated cisplatin form, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ [7,22,23,29]. However, these rate constants correspond to values averaged over all the reacting guanines, and do not provide any clues to the sequence-selectivity of the first DNA-binding step. We have therefore initiated a program aiming at the measurement of the second-order rate constants characterizing the coordination of individual guanines with the aquated cisplatin species, *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**1**) and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**2**). For this purpose, we have been using oligonucleotides bearing only one or a few guanines, allowing their specific platination rates to be resolved. Two problems have to be dealt with in such measurements. First, in sequences that allow the platinum monoadduct(s) to convert to a (several) diadduct(s) (e.g. the GG/CC sequence), this monoadduct-to-diadduct conversion overlaps in time with the platination reaction(s). Secondly, in oligonucleotide sequences forming more than one monoadduct (e.g. GG/CC; GC/GC), the isomeric monoadducts that differ only in the position of platinum binding have to be individually quantified, which sets high demands on the separation technique.

We have developed a method consisting in blocking the reaction in samples withdrawn at different time intervals by addition of KCl or KBr in excess and cooling to liquid nitrogen temperature, and analyzing subsequently the quenched samples by means of HPLC [66]. The initial, intermediate, and final products are isolated using preparative HPLC columns and identified by means of mass spectroscopy [19–21] or by co-elution with authentic samples [66,69]. This procedure yields the time-dependent concentration of the starting oligonucleotide, monoadduct(s), and diadduct(s) (when formed). The method was tested on a series of GG-containing single-stranded oligonucleotides reacting with **2** [66,69] and $[Pt(NH_3)_3(H_2O)]^{2+}$ (**3**) [20,68,69], and subsequently applied to the platination of the self-complementary duplex $d(TTGGCCAA)_2$ [19]. The use of a palindromic sequence was thought to simplify the HPLC separation (which is carried out under denaturing conditions) but has complicated somewhat the analysis (i) since the presence of two equivalent GG groups per duplex enhances the reactivity of each guanine by an unknown factor between 1 and 2, and (ii) because of the possibility of a monoadduct to be platinated by a second platinum entity on the other strand. We therefore decided to carry out the final comparative study on the series of non-palindromic, hairpin-stabilized duplexes shown in Chart 1. These oligonucleotides were reacted with the two aquated cisplatin forms, **1** and **2**, as well as with **3**; comparison of rate constants determined for these three complexes allowed us to study the impact of the nature of the platinum ligands on the reaction kinetics.

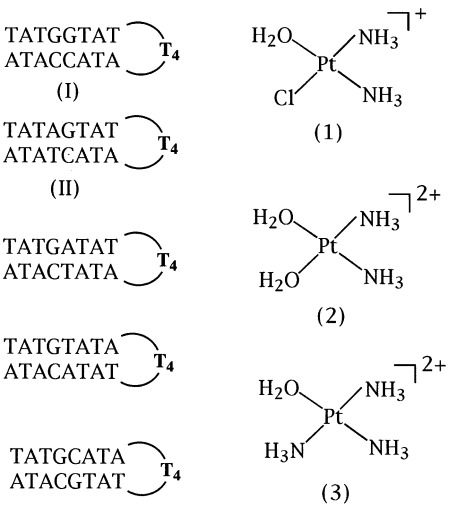
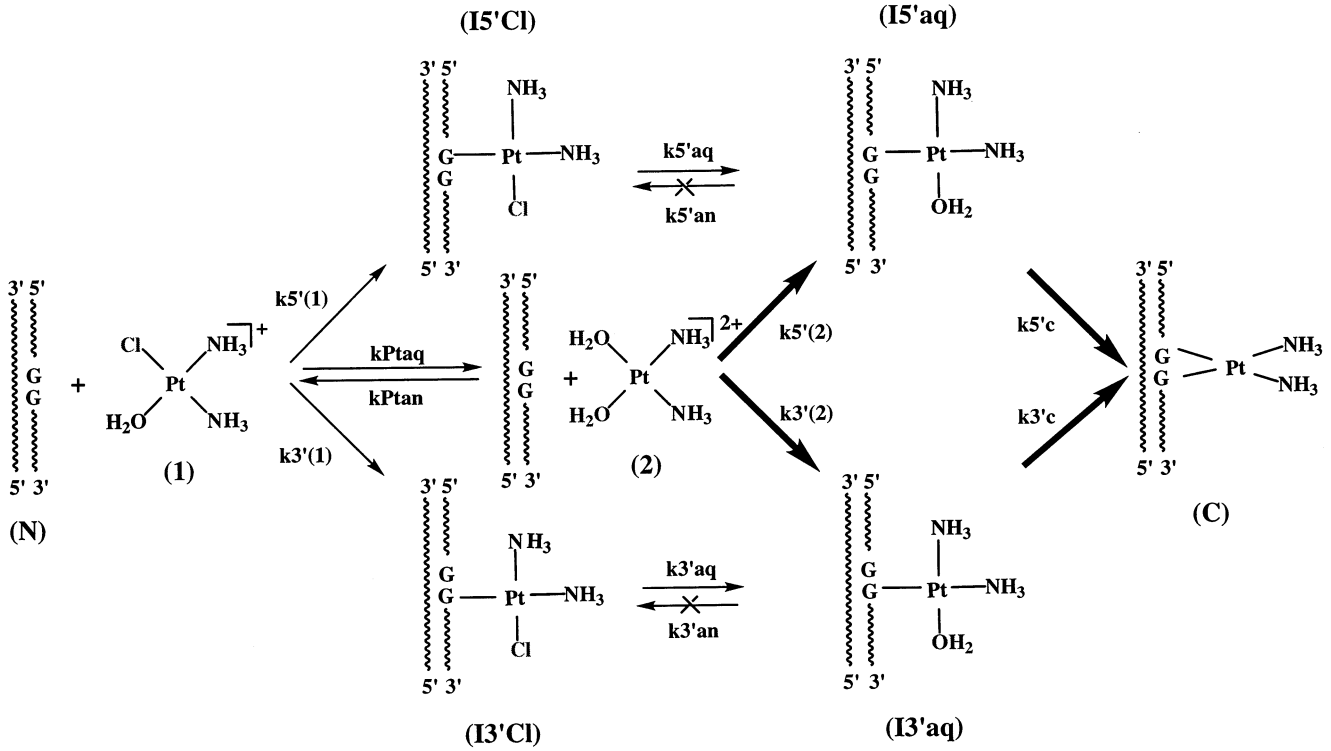


Chart. 1

In the first part of our program, we have compared the reactivity of the GG- and AG-containing duplex oligonucleotides **I** and **II** towards the platinum complexes **1**, **2**, and **3** (Chart 1). The melting points of the hairpin-duplex forms of **I** and **II** lie at 55 and 46.5°C in 0.1 M $NaClO_4$, respectively [21]. By analogy to other hairpin-duplex structures involving a T_4 loop [79–81], the duplex stems of **I** and **II** are expected to show B-DNA-like structures in solution. Whereas the reactions with **2** can be carried out in absence of chloride, without an intervention of species **1**, the reactions of **1** always involve the two possibilities of (i) direct interaction of **1** with the duplex and (ii) a solvent-assisted pathway via hydrolysis to **2** (Scheme 2). In order to resolve this complicated reaction system, we first investigated the interaction of **I** (or **II**, respectively) with **2**, and determined the rate constants corresponding to the bold arrows in Scheme 2. Subsequently, the reaction of **I** (or **II**) with **1** was analyzed. The rate constants for the reactions involving **2** determined in the first step (bold arrows) as well as those for the reversible hydrolysis of **1** [38,39] were considered as known and the remaining rate constants (i.e. those for the second-order reactions between **1** and either purine of the duplex, $k_{5'}$ (**1**) and $k_{3'}$ (**1**), and for the hydrolysis reactions of the chloro-monoadducts, $k_{5'}$aq and $k_{3'}$aq) were evaluated by fitting calculated concentration curves to the experimental ones. These fitted curves represented the concentrations of the starting oligunucleotide, those of the 5′- and 3′-monoadducts (for each, the sum of the chloro and aqua form was quantified), and that of the final chelate. The chloride anation reactions along k_5'an and k_3'an (Scheme 2) could be neglected in our experimental conditions. In all experiments, pH was kept at 4.6 ± 0.2, so that the deprotonation of the aqua ligands in **1** ($pK_a = 6.41 \pm 0.04$ [82]) and in **2** ($pK_{a_1} = 5.37 \pm 0.09$; $pK_{a_2} = 7.21 \pm 0.09$ [82]) could be neglected. Table 1 lists the rate constants for the platination of the duplexes **I** and **II** with the platinum complexes **1**, **2**, and **3**.


(I5'Cl)
(I5'aq)
(N)
(1)
(2)
(C)
(I3'Cl)
(I3'aq)
k5'(1)
k3'(1)
kPtaq
kPtan
k5'aq
k5'an
k3'aq
k3'an
k5'(2)
k3'(2)
k5'c
k3'c
3' 5'
5' 3'
NH3
Pt
Cl
OH2
H2O
2+

Scheme 2.

Table 1
Rate constants (M^{-1} s^{-1}) for the platination of the hairpin-duplexes **I** and **II** with the platinum complexes $[Pt(NH_3)_3(H_2O)]^{2+}$ (**3**), *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**2**), and *cis*-$[PtCl(NH_3)_2(H_2O)]^{+}$ (**1**) at 293 K in 0.1 M $NaClO_4$, pH 4.6 ± 0.2, with S.D. in parentheses

	cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**2**)		$[Pt(NH_3)_3(H_2O)]^{2+}$ (**3**)		*cis*-$[PtCl(NH_3)_2(H_2O)]^{+}$ (**1**)	
	$k_{5'}$	$k_{3'}$	$k_{5'}$	$k_{3'}$	$k_{5'}$	$k_{3'}$
TATGGTAT ATACCATA **T4**	18(2)	15(1)	1.7(2)	0.8(1)	0.12(1)	0.28(1)
(I)						
TATAGTAT ATATCATA **T4**	1.5(3)	9(1)			0.08(1)	0.19(1)
(II)						

The gradual decrease in reactivity along the series **2** > **3** > **1** (Table 1) correlates with the hydrogen bonding donor capacity of the varying ligand, which is high in the case of H_2O, less significant for NH_3 [83], and null for Cl. The smaller positive charge of **1** could also contribute to its lower reactivity; however, the 10–20-fold reactivity increase between **2** and **3** (both dicationic) indicates that charge–charge interactions play rather a minor role. Hydrogen bonding between platinum ligands and the O6 atom of guanine is in fact expected to favor the interaction between a platinum complex and a guanine [84–86]. Such a mechanism should not operate in the case of adenine binding, where the C6 carbon bears an amino group. Indeed, we observe for the AG adenine of **II** a considerably smaller drop in platination rate between **2** and **1** (ca. one order of magnitude) than for the 5′-guanine of **I** (ca. two orders of magnitude). In addition to this first order effect due to the interaction with the platinated base, there is apparently a weak second-order effect implicating the neighboring bases, as indicated by the fact that the ratio $k_{5'}$ (**3**)/$k_{5'}$ (**2**) is slightly different from $k_{3'}$ (**3**)/$k_{3'}$ (**2**). Such a ligand effect on the platination selectivity has also been observed for other single- and double-stranded oligonucleotides [19,69]. The presence of two leaving aqua ligands on **2** probably contributes to some extent to the very high reactivity of this species.

Table 1 allows one to compare how **1** and **2** differentiate between the GG and AG sequences of **I** and **II**, respectively. Whereas **2** reacts with both guanines of **I** ca. twice as fast as with that of **II**, the reactivity of **1** towards all the guanines of **I** and **II** appears similar. Moreover, the adenine of **II** reacts with **1** with a rate constant close to that of a guanine. Comparing the global platination rates of the GG sequence in **I** with those of the AG sequence in **II**, we observe for **2** a 3:1 preference for GG over AG, whereas **1** favors GG only slightly over AG.

The rate constants observed for the platination of **I** by **1** may be compared to those measured by Berners-Price et al. for the reaction between the double-stranded

14-mer d(ATACATG*G*TACATA)–d(TATGTACCATGTAT) and **1** in 9 mM sodium phosphate at pH 6 and 25°C using [^{1}H,^{15}N]-NMR spectroscopy: $k_{3'} = 0.54 \pm 0.02\ M^{-1}\ s^{-1}$; $k_{5'} = 0.20 \pm 0.01\ M^{-1}\ s^{-1}$ [55] (the assignment of the rate constants was achieved in a subsequent HPLC study [56]). These values are, however, approximate since they do not take into account the deprotonation of **1** at pH 6 and the hydrolysis of **1** to **2**.

Table 1 shows that the preferential binding of **2** to GG is due to the enhancement of the rate constants for both the 5′-G and the 3′-G of **I**, which are platinated with similar rates. This result is in contrast to the 12-fold 5′-G over 3′-G preference observed for the binding of **2** to d(TTGGCCAA)$_2$ under the same reaction conditions ($k_{5'} = 54 \pm 7\ M^{-1}\ s^{-1}$; $k_{3'} = 4.4 \pm 0.7\ M^{-1}\ s^{-1}$) [19]. This difference may be due to a particular structure of the palindrome d(TTGGCCAA)$_2$, as suggested by an NMR analysis [87]. The dependence of the platination kinetics on the particular local structure is clearly an issue that has to be addressed in future work.

3.2. Monoadduct-to-diadduct conversion: sequence dependence

The decay of Pt–DNA monoadducts bearing one Cl^- ligand follows first order kinetics, with a rate constant of $4–9 \times 10^{-5}\ s^{-1}$ at 37°C [7–9]. These rate constants are of the same order of magnitude as those determined for the hydrolysis of the chlorotriamine complexes $[PtCl(NH_3)_3]^+$ ($1.1 \times 10^{-5}\ s^{-1}$) [42], [PtCl-(dien)]$^+$ ($6.5 \times 10^{-5}\ s^{-1}$) [42], and *cis*-$[PtCl(NH_3)_2(dGuo)]^+$ ($1.3 \times 10^{-5}\ s^{-1}$) [51] (all at 20°C). The hydrolysis of the second chloride ligand appears thus to be the rate-limiting step in the conversion of the chloro-monoadducts to diadducts. Leng

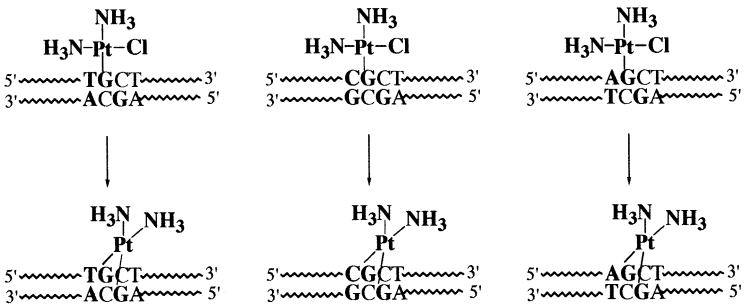


Fig. 1. Conversion of monoadducts to interstrand diadducts and the corresponding first-order rate constants (taken from Ref. [18]). The extrapolation of the rate constants to 20°C was based on the assumption that the rate constant is proportional to $2^{T/10}$.

et al. isolated monoadducts with a *cis*-$PtCl(NH_3)_2^+$-unit bound to a unique guanine within several double-stranded 22-mers, and measured their rates of conversion to diadducts [18]. Fig. 1 shows three of these monoadducts, differing only in the base-pair involving the base adjacent to the monoadduct from the 5′-side, together with the corresponding conversion rate constants. The observation that chloride arrests the monoadduct-to-diadduct conversion [18] suggests that the conversion passes via a hydrolysis to aqua-monoadducts. From Fig. 1, it can thus be concluded that the 5′-neighbor has a non-negligible influence on the rate-determining hydrolysis step. Another indication showing that the chloride hydrolysis of platinum monoadducts is sequence-dependent could be extracted from the work of Berners-Price et al., in which the two *cis*-$[PtCl(NH_3)_2G^*]^+$ monoadducts of the duplex d(ATACATG*G*TACATA)–d(TATGTACCATGTAT) (**III**) were found to decay with the global rate constants of 0.7×10^{-5} s^{-1} (5′) and 4.6×10^{-5} s^{-1} (3′) at 25°C [55,56]. Since the chelation of the corresponding aqua monoadducts was observed to be one order of magnitude faster [56], these global rate constants must mainly reflect the hydrolysis of the chloro ligands. In an effort to explain the sequence-dependence of the monoadduct hydrolysis, Kozelka and Barre hypothesized that the monoadduct-to-diadduct conversion might be catalyzed by the phosphodiester group situated at the 5′-side of the monoadduct, postulating a guanine–platinum–phosphodiester macrochelate as a possible intermediate [88]. They were able to show that such a macrochelate is indeed formed upon the reaction between *cis*-$[Pt(NO_3)_2(NH_3)_2]$ and d(TpG) in *N*,*N*-dimethylformamide. However, as we have recently determined, the hydrolysis rate of *cis*-$[PtCl(NH_3)_2$(5′-Me-dGMP)] (5′-Me-dGMP is the methyl ester of 5′-dGMP), 0.9×10^{-5} s^{-1}, is similar to that of *cis*-$[PtCl(NH_3)_2(dGuo)]^+$, 1.3×10^{-5} s^{-1} (both at 20°C) [51]. This result does not lend any support to the phosphodiester-catalysis proposal.

Recently, we were able to determine the hydrolysis rates for the monoadducts bearing a *cis*-$PtCl(NH_3)_2^+$ residue on either guanine of the hairpin-duplex **I** (see Section 3.1). The rate constants are 0.17×10^{-5} s^{-1} (5′) and 1.9×10^{-5} s^{-1} (3′) at 20°C [89]. The comparison with the hydrolysis rate constants for *cis*-$[PtCl(NH_3)_2$(5′-Me-dGMP)] (0.9×10^{-5} s^{-1}) and *cis*-$[PtCl(NH_3)_2(dGuo)]^+$ (1.3×10^{-5} s^{-1}) measured under the same conditions [51] clearly suggests that the chloride hydrolysis of the 5′-monoadduct of **I** is inhibited. The origin of this inhibition is so far unclear. It is noteworthy that in this case, as well as in the examples of sequence-dependent monoadduct hydrolysis given above [18,55,56], the monoadduct with an adjacent 5′-thymine is hydrolyzed more slowly than the other monoadducts. Does a 5′-thymine have an inhibitory effect on the hydrolysis of chloro-monoadducts? Interesting in this context is the observation made by Berners-Price et al., that the ^{15}N chemical shift of one of the $Pt–NH_3$ ligands in the 5′-chloro-monoadduct of **III** (i.e. in the more slowly hydrolyzing monoadduct) markedly differs from that measured for the analogous single-stranded monoadduct [55,56]. The authors argued that this may arise from specific interactions between the Pt ligands and the duplex which would stabilize the chloro-monoadduct and account for its long lifetime. Using molecular modeling, we have attempted to identify the molecular basis for such a stabilization within a TG*/CA dinucleotide

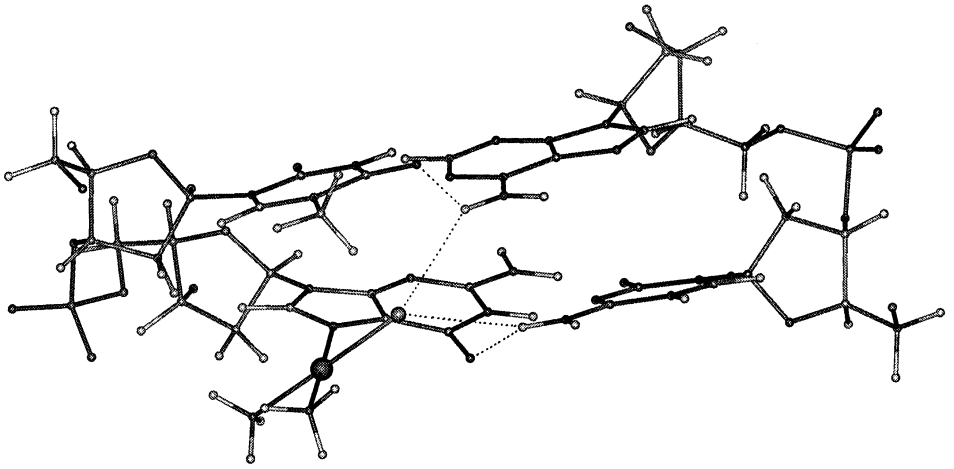


Fig. 2. Tentative hydrogen bonding scheme for a d(TG*)–d(CA) dinucleotide within a B-DNA double helix, with a *cis*-$PtCl(NH_3)_2^+$-moiety bound to G*.

(G* is bearing a *cis*-$PtCl(NH_3)_2^+$ residue) embedded in a B-DNA duplex. Fig. 2 shows that a slight propeller-twisting of both base pairs within TG*/CA could give rise to a zigzag hydrogen bonding pattern, with participation of the chloro ligand of platinum. The stability of this structural element is currently being tested by means of a molecular dynamics simulation.

3.3. Sequence-dependence of the chelating step

Table 2 shows the rate constants for chelation of the aqua-monoadducts formed on GG and AG sequences within single- and double-stranded oligonucleotides. The upper part of Table 2 groups results obtained under strictly identical conditions, i.e. $T = 293.1 \pm 0.2$ K, pH = 4.5 ± 0.1, 0.1 M $NaClO_4$. The bottom part lists experiments carried out at a lower temperature (288 ± 0.2 K) and a slightly higher pH (initial: 4.75–4.9; final: 5.2 for the HPLC study and unknown for the NMR studies). These differences account, at least partly, for the generally lower rate constants observed in the bottom part of Table 2.

Two general trends can be identified upon perusal of Table 2. First, chelation is slower in duplexes than in single strands of comparable length. Second, the differentiation between $k_{5'c}$ and $k_{3'c}$ is more pronounced in duplexes (generally one order of magnitude in favor of $k_{3'c}$) than in single-strands. At first glance, the faster chelation of the 3′-monoadduct in a B-DNA duplex could be thought related to the shorter distance separating the platinum atom from the N7 atom of the 5′-guanine, compared to the distance between the atoms N7(3′-G) and Pt in the 5′-monoadduct [19,90]. However, for $d(TTGGCCAA)_2$, we observed that the 3′-monoadduct forms an interstrand GG crosslink significantly faster ($k_{3'i} = 0.4\ s^{-1}$) than reacts the 5′-monoadduct to yield an intrastrand chelate ($k_{5c} = 0.06\ s^{-1}$), although the Pt–N7 separation is almost twice as long for the interstrand chelation [19]. In fact, two

Table 2
Rate constants for the chelation of oligonucleotide monoadducts bearing a *cis*-$Pt(NH_3)_2(H_2O)^{2+}$ residue on a GG or an AG segment

	Intrastrand chelation		Interstrand chelation	Method	Ref.
	$k_{5'c}$ (10^{-3} s^{-1})	$k_{3'c}$ (10^{-3} s^{-1})	$k_{3'i}$ (10^{-3} s^{-1})		
d(**GG**)[a]	1.5(1)	2.1(2)		HPLC	[69]
d(**TGG**)[a]	1.2(1)	1.7(2)		HPLC	[66]
d(**TTGG**)[a]	1.3(1)	3.9(7)		HPLC	[59]
d(CT**GG**CTCA)[a]	1.0(3)	3.3(4)		HPLC	[59]
d(TAT**GG**TAT)[a]	1.0(1)	4.1(2)		HPLC	[89]
d(**TTGG**CCAA)$_2$[a]	0.06(4)	0.8(2)	0.4(1)	HPLC	[19]
d(AAC**GG**TTAA–CCGTTAATT)$_2$[a]	0.41(1)	4(1)		HPLC	[96]
	0.18(5)	1.9(1)		HPLC	[21]
TATGGTAT ATACCATA **T_4** **(I)**[a]	0.3(1)	0.08(1)		HPLC	[21]
TATAGTAT ATATCATA **T_4** **(II)**[a]					
d(ATACAT**GG**TACATA)[b]	0.086(3)	0.44(5)		NMR	[56]
	0.060(5)	0.28(5)		HPLC	[56]
d(ATACATG*G*TACATA)–d(TATGTACCATGTAT) (**III**)[b]	0.049(4)	0.25(3)		NMR	[56]

[a] 293.1 ± 0.2 K, 0.1 M $NaClO_4$, pH 4.5 ± 0.1, with S.D. in parentheses.
[b] 288.0 ± 0.2 K, 0.1 M $NaClO_4$, initial pH 4.8 ± 0.1, with S.D. in parentheses.

factors determining the chelation rate constants are not included in the simple distance considerations on static models: (i) the different nucleophilic powers of the chelating N7 atoms [19], and (ii) the flexibility of the monoadduct, whose influence is apparent from the generally faster chelation rates in single strands compared to duplexes. Molecular dynamics simulations of the solvated monoadducts might enable us in the future to predict relative collision probabilities between the reacting atoms and thus to evaluate—at least semiquantitatively—relative chelation rate constants.

3.4. Which cisplatin form interacts with DNA in vivo?

According to a widely accepted view, the major DNA-binding cisplatin form in vivo is the monoaquamonochloro complex **1** [28]. Support for this assumption came from the experiments by Bancroft et al., who reacted fragments of chicken erythrocyte DNA (455 mM in nucleotides) with *cis*-$[PtCl_2(NH_3)_2]$ or **1** (32 mM) in 3 mM chloride, and detected chloro-monoadducts with DNA but no aqua-monoadducts [7]. In these in vitro conditions with a high concentration of relatively short DNA fragments, **1** is apparently the main reacting species. Johnson et al. platinated calf thymus DNA of varying nucleotide concentrations with **1** and showed that in diluted DNA solutions, the pathway via aquation to **2** is important, estimating that in DNA 0.1 mM in nucleotides, 60% of **1** reacted directly and 40% lost a chloride and bound to DNA as **2** [29]. Clearly, the distribution between these two pathways in vitro depends on the concentrations of both DNA and chloride.

In a cell nucleus, the situation is quite different from that in a diluted aqueous solution, and it is not sure whether it can be modeled by in vitro experiments. Moreover, the concentrations in the cytoplasm do not reflect the species distribution around nuclear DNA. This has become particularly clear after Jennerwein and Andrews reported that depletion of intracellular chloride to ca. 8% of the normal level does not measurably affect the platination of nuclear DNA [91]. Their observations suggest that the (eventual) establishment of an equilibrium between the different cisplatin forms in the cytoplasm is quite irrelevant to what happens on the DNA surface in the nucleus.

One possibility of tackling the problem resides in attempting to obtain indirect indications about the species reacting with DNA in vivo from the comparison of the site-selectivity determined for the individual cisplatin forms in vitro with that observed for the DNA platination in vivo. The latter yields, as stated in Section 3.1, 60–65% of GG and ca. 20% AG diadducts. The selectivity of which species do these percentages reflect? As discussed in Section 3.1, **1** binds to the GG sequence of duplex **I** and to the AG sequence of duplex **II** with comparable rates (Table 1). This result suggests that if **1** were the major species reacting with DNA in vivo, one should find similar amounts of GG and AG adducts. Moreover, since the adenine of **II** was found to react with **1** to a non-negligible extent, and the adenine-bound chloro-monoadduct was observed to undergo a relatively slow hydrolysis ($k = 0.26 \times 10^{-5}$ s^{-1} at 20°C [89]), one would expect DNA platination with **1** to produce significant numbers of persistent platinum monoadducts bound

to adenine. Both predictions are in obvious contradiction with known in vivo data [14,74,75]. It appears therefore rather unlikely that **1** be the major species reacting with DNA in vivo.

An additional indication that **1** is not likely to be the major species reacting with DNA in vivo has emerged from a generally ignored experiment by Johnson and Butour [92]. The authors compared the reaction of an equilibrated cisplatin solution containing mainly **1** with two DNAs having different G–C contents: DNA from *Micrococcus lysodeikticus* (35% G) and DNA from *Clostridium perfringens* (15.8% G). Using DNA in excess and short reaction times which did not allow the hydrolysis of **1** to proceed to a significant degree, they determined the rate of platinum binding for both DNAs. The ratio between the rate of platination of *M. lysodeikticus* DNA and that of *C. perfringens* DNA (determined under identical conditions) was 1.7 ± 0.7, which agrees within experimental error with the value of 2.2 expected for single guanines as binding sites, whereas for GG pairs as binding sites, the theoretically predicted ratio is 4.3. Thus, in this in vitro experiment where the species interacting with DNA obviously is **1**, GG sequences apparently were not preferentially platinated.

At physiological pH, both **2** ($pK_{a_1} = 5.4$; $pK_{a_2} = 7.2$) and **1** ($pK_a = 6.4$) [82] will undergo protolytic dissociation, yielding *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ and *cis*-$[PtCl(OH)(NH_3)_2]$, respectively. Whereas the reactivity of *cis*-$[PtCl(OH)(NH_3)_2]$ towards DNA is expected to be negligible, *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ still has an H_2O leaving group and thus can undergo substitution reactions. Guo et al. investigated the reaction of **2** with the octanucleotide $d(TTGGCCAA)_2$ at two different pH values and determined a reactivity ratio of 8 between *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ and **2** [93]. This value is similar to the ratio of 10 found between the reactivities of **2** and *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ towards inosine [94]. On the other hand, Johnson et al. did not observe any reduction of reaction rate between calf thymus DNA and **2** upon the first deprotonation of the platinum complex [29]. All these results point to a considerable reactivity of *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$. Even if *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$ reacts with DNA 10 times more slowly than **2**, it still remains considerably more reactive than the chloroaqua complex **1**, which is 20–150 times less reactive than **2** towards the individual base of the oligonucleotides **I** and **II** (Table 1), and, according to Johnson's estimate [29], 500 times less reactive than **2** towards calf thymus DNA. These reactivity and availability considerations further strengthen the conjecture that the couple *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$/*cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, rather than *cis*-$[PtCl(NH_3)_2(H_2O)]^+$, may be the major species reacting with DNA in vivo.

4. Conclusion

When a nucleoside is integrated into the framework of a DNA double-helix, its reactivity is modulated in a sequence-dependent manner. Thus, the reactivity of a DNA deoxyguanosine towards a platinum complex such as *cis*-$[PtCl(NH_3)_2(H_2O)]^+$ (**1**) or *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (**2**) is dependent on the neigh-

boring residues. Similarly, the kinetics of the reactions undergone by a platinum–DNA monoadduct depend on the flanking nucleotides. The present article has focused on oligonucleotide studies conceived to shed light on this sequence-dependence. Rather unexpectedly, our kinetic measurements on the reactions between hairpin-stabilized duplex oligonucleotides and platinum complexes have indicated that the general assumption, according to which the monoaquated cisplatin form **1** is the main species reacting with DNA in vivo, may be incorrect. Thus, cisplatin might undergo hydrolysis of both chloro ligands before reacting with nuclear DNA. Jestin et al. recently suggested that the second aquation step could occur within the outersphere complex formed between **1** and DNA [77]. The surface of DNA is in fact known to be depleted of anions [95], which should favor hydrolysis. Further investigations of this issue will include the evaluation of the binding preferences of the deprotonated form of **2**, *cis*-$[Pt(OH)(NH_3)_2(H_2O)]^+$, which presently appears to be the best candidate for the species interacting with nuclear DNA.

Cisplatin–DNA monoadducts are reactive intermediates with the potential to form DNA–Pt–protein crosslinks. The probability that a DNA–Pt–protein crosslink is formed depends primarily on the lifetime of the monoadduct with respect to its conversion to a diadduct with DNA. The lifetime of chloro-monoadducts, which are formed by coordination of a DNA guanine to **1**, is determined by the rate of chloride hydrolysis. Aqua-monoadducts, formed either by hydrolysis of chloro-monoadducts, or by a guanine coordination to **2**, are still fairly long-lived, as indicated by oligonucleotide studies. If we extrapolate the lifetimes observed for duplex GG-containing oligonucleotides at 20°C (Table 2) to the body temperature of 37°C, we obtain half-life times of between 1 min and 1 h. 5′-Aqua-monoadducts have generally one order of magnitude longer lifetimes than 3′-aqua-monoadducts; this is one major finding of these oligonucleotide studies. The other important result is the observation that the lifetime of the chloro-monoadducts is also sequence-dependent. Future studies will have to focus on the mechanism of the monoadduct rearrangements; it can be expected that they will bring us new insights into possible catalytic pathways for reactions on metal centers bound to DNA.

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