Energetics, Conformation, and Recognition of DNA Duplexes Modified by Methylated Analogues of [PtCl(dien)]^+**


Abstract: In early studies of empirical structure–activity relationships, monodentate PtII complexes were considered to be biologically inactive. Examples of such inactive monodentate PtII compounds are [PtCl(dien)]^+ (dien = diethylenetriamine) and [PtCl(NH_3)_3]^+. DNA is considered the major biological target of platinum compounds. Thus, monodentate DNA binding of PtII compounds was previously expected to display insignificant biological effects because it was assumed to affect DNA conformation and downstream cellular processes markedly less than the cross-links of bifunctional PtII complexes. More recently it was shown that some monodentate PtII complexes do exhibit biological effects; the active monodentate PtII complexes commonly feature bulkier amine ligands than the hitherto used dien or NH_3 groups. We were therefore interested in determining whether a simple but marked enhancement of the bulkiness of the dien ligand in monodentate [Pt(NO_3)(dien)]^+ by multiple methylation of this ligand affects the early phases in which platinum compounds exert their biological activity. More specifically, the goals of this study, performed in cell-free media, were to determine how the modification of DNA duplexes by methylated analogues of [Pt(NO_3)(dien)]^+ affects their energetics and how the alterations of this biophysical parameter are reflected by the recognition of these duplexes by DNA polymerases and the DNA repair system. We have found that the impact of the methylation of [Pt(NO_3)(dien)]^+ on the biophysical properties of DNA (thermodynamic, thermal, and conformational properties) and its biochemical processes (DNA polymerization and the repair of DNA adducts) is remarkable. Hence, we conclude that monodentate DNA binding of PtII compounds may considerably affect the biophysical properties of DNA and consequently downstream cellular processes as a result of a large increase in the bulkiness of the nonleaving ligands in this class of metal complex.

Keywords: DNA · DNA recognition · DNA structures · platinum · polymerization

Introduction

Monofunctional PtII compounds, such as [PtCl(dien)]Cl (dien = diethylenetriamine = 1,4,7-triazahexeptane; Figure 1A)

Figure 1. Structures of the platinum compounds used in this work: A) [PtCl(dien)]^+. B) [Pt(NO_3)(dien-Me_3)]^+. C) [Pt(NO_3)(dien-Me_5)]^+. D) cisplatin.

or [PtCl(NH_3)_3]Cl, have been frequently used in the studies of mechanisms underlying the biological effects of bifunctional antitumor PtII drugs such as cisplatin (Figure 1D), carboplatin, and oxaliplatin as model compounds. These monofunctional compounds have made it possible to simulate and examine the first step of binding of bifunctional PtII compounds to DNA, which is considered the major pharma-
Results

DNA binding in a cell-free medium: The rate of binding of [PtCl(dien)]$^+$ and its methylated analogues to double-helical calf thymus (CT) DNA was determined at an $r_i$ of 0.04 and 0.1 ($r_i$ is defined as the molar ratio of the free platinum complex to nucleotides at the onset of incubation with DNA) in 10 mM NaClO$_4$ at 37°C in the dark. After 24 h, an aliquot of the reaction mixture was withdrawn and assayed by differential pulse polarography (DPP) for platinum not bound to DNA. After 24 h of reaction, the binding of [PtCl(dien)]$^+$ and [Pt(NO$_3$)(dien-Me$_3$)]$^+$ was complete. A consequence of more extensive methylation, such as that in the pentamethylated analogue [Pt(NO$_3$)(dien-Me$_5$)]$^+$, was that the binding of this analogue was not quantitative; 83% of the pentamethylated complex was bound after 24 h.

The binding experiments carried out in this work indicated that modification reactions resulted in the irreversible coordination of the Pt$^{II}$–dien compounds, which thus facilitated sample analysis. Hence, it was possible to prepare samples of DNA modified by Pt$^{II}$–dien compounds at a preselected value of $r_i$ ($r_i$ values are defined as the number of atoms of metal bound per nucleotide residue). Thus, unless stated otherwise, samples of DNA modified by Pt$^{II}$–dien compounds and analyzed by biophysical or biochemical methods were prepared in NaClO$_4$ (10 mM) at 37°C. After the reactions of DNA with the complexes for 24 h, the samples were precipitated in ethanol, dissolved in the medium necessary for a particular analysis, and the $r_i$ values of aliquots of the samples were determined by flameless atomic absorption spectrophotometry (FAAS). In this way, all analyses described in this paper were performed in the absence of unbound (free) Pt$^{II}$–dien complex.

The preferential DNA binding sites of the monofunctional complexes [Pt(NO$_3$)(dien-Me$_5$)]$^+$ and [Pt(NO$_3$)(dien-Me$_3$)]$^+$ were determined by transcription mapping.$^{[17,18]}$ pSP73KB DNA contained T7 RNA polymerase promoter (part of the nucleotide sequence of this plasmid used for mapping is shown in Figure 2B). In vitro RNA synthesis by the action of RNA polymerases on this DNA template containing cross-links of several bifunctional Pt$^{II}$ compounds can be prematurely terminated at the level or in the proximity of the cross-links.$^{[17–28]}$ Interestingly, monofunctional DNA adducts of some platinum complexes, such as [PtCl(dien)]$^+$ or [PtCl(NH$_3$)$_2$]$^+$, are unable to terminate RNA synthesis.$^{[17–21]}$ Importantly, in contrast to [PtCl(dien)]$^+$, its methylated analogues, such as [Pt(NO$_3$)(dien-Me$_5$)]$^+$ and [Pt(NO$_3$)(dien-Me$_3$)]$^+$, formed DNA adducts that efficiently terminate RNA synthesis (Figure 2A).

The major stop sites were roughly identical for both methylated Pt$^{II}$–dien complexes and their profiles are similar to that obtained for DNA treated with the anticancer drug cisplatin (lane cisPt in Figure 2A). The major stop sites for DNA modified by [Pt(NO$_3$)(dien-Me$_5$)]$^+$, [Pt(NO$_3$)(dien-Me$_3$)]$^+$, and cisplatin are shown in Figure 2B. Thus, these results suggest that the major sites in DNA at which the monofunctional complexes [Pt(NO$_3$)(dien-Me$_5$)]$^+$ and [Pt(NO$_3$)(dien-Me$_3$)]$^+$ preferentially bind are guanine residues.

Differential scanning calorimetry (DSC): A calorimetric technique was used to characterize the influence of the monofunctional adduct formed by Pt$^{II}$–dienPt complexes on the thermal stability and energetics of the site-specific platinum 15-base-pair (bp) DNA duplexes (their nucleotide sequences are shown in Figure 3D). Such thermodynamic data can reveal how the platinum adduct influences duplex stability, a property that has been shown to play a significant role in the mechanism of biological activity of platinum antitumor drugs.$^{[22–27]}$ In this work we studied oligodeoxycytidinu
cleotide duplexes containing unique monofunctional adducts formed by [PdCl(dien)]^+, [Pt(NO_3)(dien-Me_3)]^+, and [Pt(NO_3)(dien-Me_5)]^+ complexes at guanine residues in three different sequence contexts, TGT, 5'-AGT, and 5'-GGG(-7-deazaG)GT. In pyrimidine-rich strands containing the central sequence 5'-GGT, 5'-G was replaced by 7-deazaguanine (7-deazaG). The 7-deazaG is an isosteric analogue of native guanine in which the aromatic N7 atom is replaced by C-H, which accurately mimics the properties of the natural base. As the guanine N7 is the site at which platinum complexes preferentially react in DNA, 7-deazaG is incapable of forming platinum adducts. Thus, 7-deazaG makes it possible to prepare a single monofunctional adduct of Pt^{II}-dienPt complexes at the central G in a 5'-GGT sequence context of pyrimidine-rich oligodeoxyribonucleotides, that is, 5'-G is 7-deaza-G.

Figure 3A–C shows DSC melting profiles (ΔC_p versus T) for the parent unmodified 15 bp duplexes TGT(15), AGT(15), and GGT(15) (solid curves) and the same duplexes containing a single monofunctional adduct of [PdCl(dien)]^+ (dotted and dashed curves), [Pt(NO_3)(dien-Me_3)]^+ (dashed curves), and [Pt(NO_3)(dien-Me_5)]^+ (dotted curves). Each transition showed negligible changes in the heat capacities between the initial and final states, and denaturation (heating) and renaturation (cooling) curves for the unmodified and platinated duplexes were superimposable (not shown), which is consistent with the reversibility of the melting equilibrium. Our calorimetric data, described below, were interpreted on the assumption that the thermodynamic parameters for the melting of the unmodified and platinated duplexes can be ascribed to differences in the initial duplex states. This implies that the final single-stranded states should be thermodynamically equivalent at the elevated temperatures at which they are formed. This assumption was verified (not shown) similarly to earlier reports by recording identical circular dichroic spectra for samples of unplatinated and platinated duplexes heated at high temperatures (90°C). Overall, meaningful thermodynamic data from the calorimetric measurements described below could be obtained.

DSC melting profiles were analyzed as described in the Experimental Section and the results are listed in Table 1. All thermodynamic parameters discussed in this work refer to the duplex dissociation process. Differences in the dissociation thermodynamics due to the presence of an adduct are
Table 1. Calorimetrically derived thermodynamic parameters for the dissociation (melting) of the 15-bp duplexes that are unmodified or contain a single, site-specific monofunctional adduct of [PtCl(dien)]\textsuperscript{+}, [Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})\textsuperscript{+}], or [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})\textsuperscript{+}].

<table>
<thead>
<tr>
<th>Adduct</th>
<th>$T_m$ [°C]\textsuperscript{a}</th>
<th>$\Delta H_{dl}$ [kJ mol\textsuperscript{-1}]\textsuperscript{b}</th>
<th>$\Delta S$ [kJ mol\textsuperscript{-1} K\textsuperscript{-1}]\textsuperscript{b}</th>
<th>$\Delta G_{dl}$ [kJ mol\textsuperscript{-1}]\textsuperscript{b}</th>
<th>$K_0$ [μM]\textsuperscript{-1}</th>
<th>$\Delta H_{dl}$ [kJ mol\textsuperscript{-1}]</th>
<th>$\Delta S$ [kJ mol\textsuperscript{-1} K\textsuperscript{-1}]</th>
<th>$\Delta G_{dl}$ [kJ mol\textsuperscript{-1}]</th>
<th>$K_0$ [μM]\textsuperscript{-1}</th>
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<tbody>
<tr>
<td>no Pt (control)</td>
<td>58.9</td>
<td>417</td>
<td>1.261</td>
<td>41</td>
<td>0.086</td>
<td>417</td>
<td>1.261</td>
<td>41</td>
<td>0.086</td>
</tr>
<tr>
<td>[PtCl(dien)]\textsuperscript{+}</td>
<td>53.0</td>
<td>386 (−31)</td>
<td>1.187 (−0.074)</td>
<td>32 (−9)</td>
<td>2.48</td>
<td>386 (−31)</td>
<td>1.187 (−0.074)</td>
<td>32 (−9)</td>
<td>2.48</td>
</tr>
<tr>
<td>[Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})\textsuperscript{+}]</td>
<td>44.5</td>
<td>307 (−110)</td>
<td>0.968 (−0.293)</td>
<td>18 (−23)</td>
<td>702.4</td>
<td>307 (−110)</td>
<td>0.968 (−0.293)</td>
<td>18 (−23)</td>
<td>702.4</td>
</tr>
<tr>
<td>[Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})\textsuperscript{+}]</td>
<td>43.9</td>
<td>304 (−113)</td>
<td>0.959 (−0.302)</td>
<td>18 (−23)</td>
<td>702.4</td>
<td>304 (−113)</td>
<td>0.959 (−0.302)</td>
<td>18 (−23)</td>
<td>702.4</td>
</tr>
</tbody>
</table>

no Pt (control)         | 58.0                          | 401                                             | 1.216                            | 38                              | 0.22  | 401                             | 1.216                            | 38                              | 0.22  |
| [PtCl(dien)]\textsuperscript{+} | 55.4                          | 371 (−30)                                       | 1.133 (−0.083)                  | 35 (−5)                         | 1.65  | 371 (−30)                       | 1.133 (−0.083)                  | 35 (−5)                         | 1.65  |
| [Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})\textsuperscript{+}] | 45.7                          | 348 (−53)                                       | 1.092 (−0.124)                  | 22 (−16)                        | 139.8 | 348 (−53)                       | 1.092 (−0.124)                  | 22 (−16)                        | 139.8 |
| [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})\textsuperscript{+}] | 46.4                          | 354 (−47)                                       | 1.110 (−0.106)                  | 23 (−15)                        | 93.46 | 354 (−47)                       | 1.110 (−0.106)                  | 23 (−15)                        | 93.46 |

no Pt (control)         | 61.1                          | 426                                             | 1.284                            | 43                              | 0.029 | 426                             | 1.284                            | 43                              | 0.029 |
| [PtCl(dien)]\textsuperscript{+} | 62.3                          | 444 (18)                                        | 1.335 (0.051)                   | 46 (3)                          | 0.087 | 444 (18)                        | 1.335 (0.051)                   | 46 (3)                          | 0.087 |
| [Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})\textsuperscript{+}] | 51.3                          | 367 (−59)                                       | 1.136 (−0.148)                  | 28 (−15)                        | 12.43 | 367 (−59)                       | 1.136 (−0.148)                  | 28 (−15)                        | 12.43 |
| [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})\textsuperscript{+}] | 51.7                          | 378 (−40)                                       | 1.170 (−0.114)                  | 29 (−14)                        | 8.31  | 378 (−40)                       | 1.170 (−0.114)                  | 29 (−14)                        | 8.31  |

[a] The $\Delta F$ and $\Delta S$ values are averages derived from three independent experiments. The experimental uncertainties of the parameters are as follows: $T_m$ (±0.5°C), $\Delta F$ (±2%), $\Delta S$ (±3%), $\Delta G_{dl}$ (±3%). The $\Delta G_{dl}$ parameters are given in parentheses (these parameters are computed by subtracting the appropriate value measured for the control, the unmodified duplex, from the value measured for the duplex containing the single, site-specific platinum adduct). [b] $K_0$ denotes the dissociation constant for strand dissociation ($\Delta G_{25}=−RT\ln K_0$; $T$ is the temperature in Kelvin, and $R$ is the universal gas constant (8.314472 J K\textsuperscript{-1} mol\textsuperscript{-1})).

DNA polymerization: It has been demonstrated that DNA modifications by various platinum complexes have signifi-

Figure 4. The contributions of enthalpic and entropic effects to the stability (free energy change) of the 15-bp duplexes TGT(15) (A), AGT(15) (B), and GGT(15) (C) containing the monofunctional adduct of [PtCl(dien)]\textsuperscript{+} (solid bars), [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})\textsuperscript{+} (open bars), or [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})\textsuperscript{+} (vertical striped bars). The units of each parameter ($\Delta H_{dl}$, $\Delta S$, and $\Delta G_{dl}$) are kJ mol\textsuperscript{-1} and $T=25$°C.

Table 1 and Figure 4, this duplex destabilization being enthalpic in origin. In this respect, the monofunctional adducts of the methylated analogues of [PtCl(dien)]\textsuperscript{+} were almost equally effective, and markedly more effective than the nonmethylated complex. Moreover, the efficiency of the adducts to enthalpically destabilize the duplex depended on the sequence context; the trend was similar to that observed for the efficiency of the adducts to reduce the DNA melting temperature (see above), that is, TGT(15) $\Delta G_{25} ≫$ AGT(15) $\Delta G_{25} ≫$ GGT(15).
cant effects on the processivity of a number of prokaryotic, eukaryotic, and viral DNA polymerases.\textsuperscript{[12–30]} Interestingly, with DNA templates containing site-specifically placed adducts of various platinum compounds, a number of prokaryotic and eukaryotic DNA polymerases were blocked, but they could also traverse through platinum adducts, depending on their character and the conformational alterations induced in DNA. It is therefore of great interest to examine whether DNA polymerases, which process DNA substrates containing monofunctional adducts of Pt\textsuperscript{II}–dien complexes, could reveal potential differences in the alterations imposed on DNA by the adducts of the three Pt\textsuperscript{II}–dien complexes, which differ in the level of methylation of the dien moiety.

In this work we investigated DNA polymerization using templates site-specifically modified by [PtCl(dien)]\textsuperscript{+}, [Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})]\textsuperscript{+}, or [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})]\textsuperscript{+} by two DNA polymerases, which differ in their processivity and fidelity. In the first series of experiments, we used the Klenow fragment of \textit{E. coli} DNA polymerase I deficient in 3'/to-5' proofreading exonuclease activity (KF\textsuperscript{+}/C\textsubscript{0}) as a model enzyme frequently used in studies aimed at understanding the processes in which nucleic acid polymerases take part.

We constructed 17-mer/30-mer (Figure 5A) primer template duplexes unplatinated or containing a monofunctional adduct of the Pt\textsuperscript{III}–dien complex formed at the guanine residue in the central TGT, 5'-AGT, or 5'-AGT-(7-deazaG)GT sequence. The first 17 nucleotides on the 3' terminus of the 30-mer template strand were complementary to the nucleotides of the 17-mer primer, and the guanine involved in the monofunctional adduct on the template strand was located 20 bases from the 3' terminus (Figure 5A). After annealing the 17-nucleotide primer to the 3' terminus of the unplatinated or platinated template strand (positioning the 3'-end of the primer three bases before the adduct in the template strand), we examined DNA polymerization by using the single monofunctional adduct of [PtCl(dien)]\textsuperscript{+}, [Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})]\textsuperscript{+}, or [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})]\textsuperscript{+} on the template by KF\textsuperscript{+} in the presence of all four deoxyribonucleoside 5'-triphosphates (dNTP). The reaction was stopped after various intervals of time and the products were analyzed by using a sequencing gel (Figure 5A).

Polymerization by KF\textsuperscript{+} using the 17-mer/30-mer primer templates containing the monofunctional adducts of the methylated Pt\textsuperscript{II}–dien complexes ([Pt(NO\textsubscript{3})(dien-Me\textsubscript{3})]\textsuperscript{+} or [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5})]\textsuperscript{+}) in the presence of all four dNTP proceeded rapidly up to the nucleotide opposite the adduct, such that the 20-nucleotide intermediate product accumulated to a significant extent (shown in Figure 5A). There was only a slight accumulation of larger DNA intermediates and full length products in particular using the templates containing the adduct of [PtNO\textsubscript{3})(dien-Me\textsubscript{3}])\textsuperscript{+}, whereas no intermediate products were seen with the 30-mer control template or the template containing the adduct of nonmethylated [PtCl(dien)]\textsuperscript{+} as the full-length product only was being formed (shown in Figure 5A). This result indicates that the character of the monofunctional adducts of the methylated Pt\textsuperscript{II}–dien complexes, such as [Pt(NO\textsubscript{3})(dien-Me\textsubscript{3}])\textsuperscript{+} or [Pt(NO\textsubscript{3})(dien-Me\textsubscript{5}])\textsuperscript{+}, and alterations induced in DNA by...
their adducts were distinctly different to those induced by the adduct of plain (nonmethylated) [PtCl(dien)]+ such that the increase in the bulkiness of the monofunctional PtII–dien complexes due to their methylation led to DNA adducts that could potentially impede the elongation of DNA.

We have also examined the effects of the monofunctional adducts of PtII–dien complexes on the polymerization by reverse transcriptase of human immunodeficiency virus type 1 (RT HIV-1) in the presence of all four dNTPs. This enzyme also possesses DNA template-dependent DNA polymerase activity, but relatively low processivity and fidelity.[39] In these studies, the elongation of the 17-mer/30-mer primer template duplexes (identical to those used in the experiments with KF− (see above)) was studied. As is shown in Figure 6, we also confirmed by using this DNA polymerase, which operates by a different mechanism to KF−, that in contrast to the monofunctional adducts of plain (nonmethylated) [PtCl(dien)]+, the adducts of its methylated analogues are also a fairly strong block to DNA synthesis catalyzed by RT HIV-1. The only substantial difference between the polymerization by KF− and RT HIV-1 was that the adducts of [Pt(NO3)2(dien-Me3)]+ represented a somewhat weaker block to DNA catalyzed by RT HIV-1 (cf. Figures 5b and 6B). In other words, the adducts of [Pt(NO3)2(dien-Me3)]+ could potentially impede the elongation of DNA by KF− to a greater extent than the elongation by RT HIV-1.

DNA repair: Figure 7 illustrates an experiment in which DNA repair synthesis by repair-proficient HeLa cell-free extract (CFE) in pSP73KB plasmid modified by cisplatin, [PtCl(dien)]+, [Pt(NO3)2(dien-Me3)]+, and [Pt(NO3)2(dienMe5)]+ at rt = 0.035 was examined. The repair activity was monitored by measuring the amount of incorporated radio-labeled nucleotide. The incorporation of radioactive material was corrected for the relative DNA content in each band. Approximately the same levels of damage-induced DNA repair synthesis were detected in the plasmid modified by cisplatin and methylated PtII–dien complexes (Figure 7A, lanes cisPt, dienPt-Me3, and dienPt-Me5, and Figure 7B). In contrast, the adducts of nonmethylated [PtCl(dien)]+ only induced a very low level of repair synthesis (approximately only 10% of that observed for repair synthesis in the plasmid modified by cisplatin; Figure 7A, lane dienPt, and Figure 7B).

Discussion

The results of the transcription mapping experiments (Figure 2) are consistent with the view that multiple methylation of the dien ligand does not affect preferential DNA binding sites of this class of monofunctional PtII complexes, that is, guanine residues. Similarly three methyl groups on the dien ligand affect DNA binding only slightly. On the other hand, the pentamethylated complex binds to DNA slightly less quantitatively after 24 h, likely due to the steric hindrance associated with the bulkiness of its nonleaving group.

DSC can provide quantitative, model-independent characterization of the effects of the lesion on duplex thermodynamics. The duplex melting temperatures (thermal stability parameter, Tm, and thermodynamic stability data, the
duplex dissociation enthalpies, $\Delta H_{\text{m}}$, and entropies, $\Delta S$, derived from analyses of the calorimetrically measured excess heat capacity, $\Delta C_p$, versus temperature profiles are listed in Table 1 along with the corresponding $\Delta G_{25}^\circ$ values calculated at 25°C. Analysis of the $T_m$ values reveals that methylation of [PtCl(dien)]$^+$ markedly enhances the efficiency of the monofunctional adducts of these Pt$^{IV}$ compounds to reduce the thermodynamic stability of DNA. However, the melting temperature is not a thermodynamic parameter. Therefore, we also examined how the introduction of the monofunctional adduct of methylated analogues of [PtCl(dien)]$^+$ affects the thermodynamic stability ($\Delta G_{25}^\circ$) of DNA duplexes.

The thermodynamic parameters derived from DSC data reveal that the two methylated complexes destabilize the double helices tested in this work significantly more than the nonmethylated [PtCl(dien)]$^+$, as indicated by a 14–23 kJ mol$^{-1}$ increase in the Gibbs free energy for duplex formation at 25°C (Table 1). Interestingly, the $\Delta \Delta G_{25}^\circ$ values observed for the melting of each duplex containing the adduct of [Pt(NO$_3$)(dien-Me$_3$)]$^+$ or [Pt(NO$_3$)(dien-Me$_5$)]$^+$ are identical or very similar (~23 kJ mol$^{-1}$ for the duplex TGT(15), ~16 or ~15 kJ mol$^{-1}$ for the duplex AGT(15) and ~15 or ~14 kJ mol$^{-1}$ for the duplex GGT(15), see Table 1), which suggests that the monofunctional adducts of the two methylated Pt$^{IV}$ analogues, the bulkiness of which is increased by three or five methyl groups, induce in DNA similar conformational alterations. Interestingly, these $\Delta \Delta G_{25}^\circ$ values represent an equilibrium preference for the unmodified duplexes over those modified by the nonmethylated [PtCl(dien)]$^+$ or the methylated [Pt(NO$_3$)(dien-Me$_3$)]$^+$ or [Pt(NO$_3$)(dien-Me$_5$)]$^+$ of, respectively, 38, 10642, or 10642 to 1 for the TGT(15) duplex, 8, 635, or 424 to 1 for the AGT(15) duplex, and ~0.3, 429, or 287 to 1 for the GGT(15) duplex. Thus, these results support the view that multiple methylation of the dien moiety in the adducts of these monofunctional complexes markedly enhances the thermodynamic destabilization of DNA, which is most pronounced if the adduct is formed in the TGT sequence. This enhanced destabilization is likely to be associated with a markedly more extensive distortion of the DNA conformation.

Figure 7. In vitro repair synthesis assay of the extract prepared from the repair-proficient HeLa cell line. Repair synthesis was performed using pSP73KB plasmid unmodified (lane noPt) or modified at $n_i = 0.035$ by cisplatin, [PtCl(dien)]$^+$, [Pt(NO$_3$)(dien-Me$_3$)]$^+$, and [Pt(NO$_3$)(dien-Me$_5$)]$^+$ (lanes cisPt, dienPt, dienPt-Me$_3$, and dienPt-Me$_5$, respectively). A) Results of a typical experiment. The top panel is a photograph of the EtBr stained gel and the bottom panel is the autoradiogram of the gel and shows the incorporation of [$c^{32}$P]dATP. B) Incorporation of dATP into unmodified or platinated plasmids. For all quantifications representing mean values of two independent experiments, incorporation of radioactive material is corrected for the relative DNA content in each band. The bars indicate the standard error of the mean (SEM).
sults from conformational alterations induced by these monofunctional adducts.

The changes in the thermodynamic stability of the duplexes examined in this work, $\Delta \Delta G_{int}$, caused by the formation of a single site-specific monofunctional adduct of the methylated analogues [Pt(NO$_3$)(dien-Me$_3$)]$^+$ or [Pt(NO$_3$)(dien-Me$_5$)]$^+$ reflect a combination of enthalpic ($\Delta \Delta H_{int}$) and entropic ($\Delta \Delta S$) effects. The magnitudes of these effects vary with the sequence context. The relative contributions of the adduct-induced changes in the enthalpy and entropy terms for the disturbance of the duplexes TGT(15), AGT(15), and GGT(15) can be seen in Figure 4. Interestingly, the differences in the transition free-energy change, $\Delta G_{int}$, observed upon formation of the monofunctional adducts of [Pt(NO$_3$)(dien-Me$_3$)]$^+$ or [Pt(NO$_3$)(dien-Me$_5$)]$^+$ are significantly smaller than the observed differences in the transition enthalpy change ($\Delta H_{int}$; Figure 4). The values of $\Delta \Delta H_{int}$ range from $-47$ to $-113$ kJ mol$^{-1}$, whereas the values of $\Delta \Delta G_{int}$ range from only $-14$ to $-23$ kJ mol$^{-1}$. Regardless of the magnitude of $\Delta \Delta H_{int}$, there is a considerable, but not complete, compensating change in the entropy term. Interestingly, the higher transition enthalpy change due to the adducts formed in the TGT sequence (compared with in the AGT or GGT sequence) is accompanied by a higher entropic compensation. The impact of the monofunctional adducts of methylated Pt$^{II}$ complexes tested in this work on the enthalpy is always destabilising, whereas the entropy term is always stabilising. The compensation does not result in invariant stability with respect to sequence context and the type of monofunctional Pt$^{II}$ complex. In addition, we have tried to answer the question whether sequence has any effect on the magnitude of the enthalpy-driven destabilization. Measured enthalpy differences of 16, $-9$, and $-25$ kJ mol$^{-1}$ for the unmodified duplexes ($\Delta H_{cal}$; TGT(15)−AGT(15), $\Delta H_{cal}$; TGT(15)−GGT(15), and $\Delta H_{cal}$; AGT(15)−GGT(15), respectively) have been found (Table 1). On the other hand, the enthalpy differences between the three duplexes (TGT(15)−AGT(15), TGT(15)−GGT(15), or AGT(15)−GGT(15)) modified by [Pt(NO$_3$)(dien-Me$_3$)]$^+$ (the values obtained for [Pt(NO$_3$)(dien-Me$_5$)]$^+$ are given in parentheses) are equal to $-41$ ($-50$), $-60$ ($-74$), and $-19$ ($-24$) kJ mol$^{-1}$, respectively (Table 1), which indicates that the monofunctional adducts formed by methylated [Pt(NO$_3$)(dien-Me$_3$)]$^+$ or [Pt(NO$_3$)(dien-Me$_5$)]$^+$ in the TGT sequence induces more extensive unstacking interactions than in the 5′-AGT or 5′-(7-deazaG)GT sequence, which leads to a greater exposure of the nonpolar surface to the solvent. This allows us to predict that inclusion of the monofunctional adduct of the methylated analogue [Pt(NO$_3$)(dien-Me$_3$)]$^+$ or [Pt(NO$_3$)(dien-Me$_5$)]$^+$ into the TGT sequence yields more pronounced conformational alterations at the site of the adduct than in the 5′-AGT or 5′-(7-deazaG)GT sequence.

The $\Delta H_{cal}/\Delta H_{int}$ ratio makes it possible to determine whether duplex-unfolding takes place in two-state transitions or through the formation of intermediates.[19] If the $\Delta H_{cal}/\Delta H_{int}$ ratio is equal to 1 then the transition takes place in an all-or-none fashion.[20] We obtained $\Delta H_{cal}/\Delta H_{int}$ ratios in the range of 1.06−1.11, which confirms that each duplex examined in this work unfolds in a two-state transition. Hence, despite affecting the thermal and thermodynamic parameters of the unfolding of the host duplexes, methylation of [PtCl(dien)]$^+$ does not affect the properties of the monofunctional adducts to the extent that they would markedly change the cooperativity of the melting transition of the host duplex. This demonstrates that neither the monofunctional adduct nor the identity of the base flanking the adduct on its 5′ site alters the ability of the duplex to propagate those interactions required for cooperative melting.

In mammalian cells, various DNA repair pathways are important mechanisms for the removal of DNA adducts, including those generated by various chemotherapeutics. For instance, efficient repair by several repair systems of various cross-links produced by bifunctional platinum drugs has been reported.[41–46] It has been suggested[47–49] that the initial recognition event of the DNA lesion repair process is dependent on the lesion-induced alterations of duplex energetics. Only a very low level of repair synthesis is noticed if DNA containing monofunctional adducts of nonmethylated [PtCl(dien)]$^+$ is used as a substrate for the DNA repair system (shown in Figure 7, lane dienPt). This observation is consistent with the view that monofunctional lesions induced in DNA by monofunctional adducts of Pt$^{II}$ compounds, such as those of [PtCl(dien)]$^+$, are not recognized by the components of DNA repair systems. Therefore we have also examined whether the enhancement of the bulkiness of monofunctional adducts of Pt$^{II}$-dien compounds by multiple methylation affects their DNA adducts to the extent that they become a substrate for DNA repair system(s). Markedly higher levels of damage-induced DNA repair synthesis are detected if DNA containing monofunctional adducts of methylated analogues are used as a substrate for the DNA repair system (Figure 7A, lanes dienPt-Me$_3$ and dienPt-Me$_5$, and Figure 7B). Thus, this markedly enhanced level of repair of the bulkier DNA adducts of methylated analogues correlates with the considerably higher thermodynamic destabilization of DNA induced by these complexes.

Similarly, monofunctional adducts of the plain and relatively small (nonmethylated) Pt$^{II}$ complexes, such as [PtCl(dien)]$^+$, are bypassed by DNA and RNA polymerases (see refs.[18,46,50,51] and Figures 5 and 6). The results of this work (Figures 5 and 6) demonstrate that in contrast to the monofunctional adducts of the small [PtCl(dien)]$^+$, the adducts of its considerably bulkier methylated analogues are a fairly strong block to DNA synthesis catalyzed by DNA polymerases. Thus, similarly to the case of DNA repair, the markedly enhanced efficiency of DNA adducts of the bulkier methylated analogues to block DNA polymerization correlates with a considerably higher thermodynamic destabilization of DNA induced by these complexes. Interestingly, there is no distinct difference between the efficiency of the adducts of [Pt(NO$_3$)(dien-Me$_3$)]$^+$ and [Pt(NO$_3$)(dien-Me$_5$)]$^+$ to reduce the thermodynamic stability of DNA and conse-
quently the DNA adducts of these different methylated complexes block DNA polymerization by KF− approximately equally (Figure 5). In contrast, the efficiency of the DNA adducts of the bulkiest pentamethylated complex to block DNA polymerization is distinctly higher than that of the less bulky [Pt(NO3)2(dien-Me5)]+ if DNA polymerization is catalyzed by RT HIV-1 (Figure 6). The different processing of the DNA adducts of the methylated complexes by KF− and RT HIV-1 may be associated with the fact that RT HIV-1 is markedly more flexible than other nucleic acid synthesizing enzymes so that it can polymerize DNA across and beyond bulker adducts more easily than other DNA polymerases. Thus, RT HIV-1, which has a sterically more flexible active site, may recognize not only the reduced thermodynamic stability of DNA and the enhanced bulkiness of DNA adducts, but, to a greater extent than other DNA polymerases, also their structure or shape.

The current models of recognition of DNA damage propose that the presence of a chemically modified nucleotide and the resulting destabilization of the duplex structure, either by the direct disruption of base-pair alignments or by more subtle perturbations, such as the unwinding or bending of the helix, are both required for DNA repair activity. The minimally perturbed structures of the duplexes modified by a relatively small molecule of [PtCl(dien)]+ and their only slightly affected thermodynamic stability are consistent with the view and the observations of this work (Figures 5–7) that the [PtCl(dien)]+ adduct is a very poor substrate for DNA repair systems and that it does not represent a strong block to DNA polymerization. On the other hand, the structures of the duplexes perturbed considerably more by the adducts of bulkier, multiply methylated [Pt(NO3)2(dien-Me5)]+ and [Pt(NO3)2(dien-Me6)]+ and the markedly reduced thermodynamic stability of these duplexes are in a excellent agreement with our results (Figures 5–7), which demonstrates that the adducts of [Pt(NO3)2(dien-Me5)]+ and [Pt(NO3)2(dien-Me6)]+ are very good substrates for DNA repair systems and represent a strong block to DNA polymerization. A common feature of various DNA lesions induced by PtII complexes, including those examined in this work, is the enthalpically driven thermodynamic destabilization of the helix. A reduction in the stability of double helices should make them susceptible to recognition of the damage. For example, less stable DNA should be more flexible and therefore should have a greater propensity to adopt the altered structure required in its productive complexes with components of DNA repair systems. So far as the catalytic efficiency of DNA polymerases is concerned, an enhanced flexibility of DNA may impede the formation of a gently tuned catalytically active structure of the ternary DNA polymerase primer/template DNA incoming dNTP complex.

The impact of methylation of [PtCl(dien)]+, which results in a considerable increase in the bulkiness of the nonleaving ligand, on the biophysical properties of DNA (such as thermodynamic, thermal, and conformational properties) and its biochemical processes (DNA polymerization and repair of DNA adducts) is remarkable (see above). Hence, it is reasonable to suggest that the bulkiness of the DNA adducts of the PtII compounds is an important parameter that controls processes associated with the biological effects of these compounds. In other words, the results of this work demonstrate that monodentate DNA binding of PtII compounds, previously expected not to play a significant role in the biological effects of these complexes, may considerably affect the biophysical properties of DNA and consequently downstream cellular processes as a result of the large increase in the bulkiness of the nonleaving ligand in this class of metal complexes. For instance, if the adducts escape repair and survive to the next round of DNA replication, error-prone translesion bypass can occur, giving rise to mutations and ultimately to cancer. In addition, the results of this work confirm that changes in the thermodynamic properties of DNA induced by its modification by PtII complexes can be used as predictors of recognition by DNA polymerases and cellular repair proteins, including the efficiency of repair mechanisms to remove the lesion from DNA and the ability of the PtII compound to block DNA polymerization catalyzed by DNA polymerases across and beyond its adduct.

It is generally accepted that DNA repair and polymerization catalyzed by DNA polymerases play an important role in downstream cellular processes following DNA damage, such as mutagenesis, carcinogenesis, and cytotoxicity. It is important to understand each of these processes individually on a molecular level to assess the potency of DNA-damaging agents to exert biological effects. The results of this work also expand the database correlating the thermodynamic characteristics of well-defined DNA damage and the susceptibility of this damage to removal by DNA repair enzymes and its efficiency to block DNA polymerization catalyzed by DNA polymerases.

**Experimental Section**

Starting materials: Cisplatin and dimethyl sulfate (DMS) were obtained from Sigma–Aldrich s.r.o. (Prague, Czech Republic). [PtCl(dien)]+, [Pt(NO3)2(dien-Me5)]+, and [Pt(NO3)2(dien-Me6)]+ (Figure 1) were prepared and characterized as described below. Stock solutions of platinum compounds for the biophysical and biochemical studies were prepared in water and stored at room temperature in the dark. The concentrations of platinum in the stock solutions were determined by FAAS. CT DNA (42% G + C, mean molecular mass ca. 2 × 107 Da) was prepared and characterized as described previously. pSP73KB (2455 bp) plasmid was isolated according to standard procedures. The synthetic oligodeoxyribonucleotides were purchased from VBC-Genomics (Vienna, Austria) and purified as described previously. Restriction endonucleases EcoRI, T4 polynucleotide kinase, and KF− were purchased from New England Biolabs. RT HIV-1 was from Amersham Pharmacia Biotech (Newport, UK). Acrylamide, bis(acrylamide), dithiothreitol, NaCN, etidium bromide (EtBr), and urea were from Merck KgaA (Darmstadt, Germany). Nonidet P-30 was from Fluka (Prague, Czech Republic). Agarose was from FMC BioProducts (Rockland, ME). Radioactive products were from MP Biomedicals, LLC (Irvine, CA). Riboprobes Genomic System II for transcription mapping containing T7 RNA polymerase was purchased from Promega (Madison, WI). A cell-free extract (CFE) was prepared from the repair-proficient HeLa S3 cell line as described previously.
Inhibition of DNA polymerization: The primer extension assays with all four dNTPs were performed with the 30-mer templates (see Figures 5A and 6A) containing a single monofunctional adduct of a Pt II–dien complex prepared as described above in the section on platination reactions. The 17-mer DNA primer (its nucleotide sequence is shown in Figures 5A and 6A) was complementary to the 3' termini of the 30-mer templates. The DNA substrates (5×10^4 M) were formed by annealing the templates and 5'-end-labeled primers in a molar ratio of 3:1. All experiments using RT HIV-1 were performed at 37°C in a volume of 50 μL in a buffer containing Tris-HCl (50 mM, pH 8.0), MgCl2 (10 mM), KCl (50 mM), dithiothreitol (3 mM), Nonidet P-30 (0.1%), dATP (100 μM), dCTP (100 μM), dGTP (100 μM), and TTP (100 μM), and RT HIV-1 (1.0 unit). The experiments with KF were performed with the same 30-mer templates and 17-mer DNA primers and the same volume, but at 25°C in a buffer consisting of Tris-HCl (50 mM, pH 7.4), MgCl2 (10 mM), dithiothreitol (0.1 mM), and bovine serum albumin (50 μg/mL); the nucleoside triphosphates were at a concentration of 100 μM and 0.5 unit of KF was used. Reactions were terminated after various intervals of time by the addition of EDTA to give a resulting concentration of 20 mM and heating at 100°C for 30 s. Products were resolved by denaturing 15% polyacrylamide/8 M urea gel and quantified by phosphor-imaging analysis. Other details have been published previously.[24–26]

DNA repair synthesis by human cell extracts: Repair DNA synthesis of CFEs was assayed by using pSP73KB plasmid. Each reaction mixture of 50 μL contained unmodified or platinated pSP73KB (500 ng), ATnP (2 μM), KCl (30 mM), creatine phosphokinase (rabbit muscle; 0.05 mg/mL), dGTP (20 μM), dCTP (20 μM), TTP (20 μM), dATP (8 μM), 74 kBq of [γ-32P]dATP in a buffer composed of HEPES-KOH (40 mM, pH 7.5), MgCl2 (5 mM), dithiothreitol (0.5 mM), creatine phosphate (22 mM), bovine serum albumin (1.4 mg/mL), and CFE (150 μg). Reactions were incubated for 3 h at 25°C and terminated by adding Na2HEDTA to give a final concentration of 20 mM sodium dodecyl sulfate to 0.6%, and proteinase K to 250 μg/mL followed by incubation for 30 min. The products were extracted with one volume of 1:1 phenol/chloroform. The DNA was precipitated from the aqueous layer by the addition of 1/50 volume NaCl (5 mM), glycogen (5 mg) and 2.5 volumes ethanol. After 20 min of incubation on ice and centrifugation at 12000 g for 30 min at 4°C, the pellet was washed with 0.5 mL 70% ethanol and dried in a vacuum centrifuge. DNA was finally linearized before electrophoresis on a 1% agarose gel containing EtBr (0.3 mg/mL). Other physical methods: Absorption spectra were measured with a Beckman 7400 DU spectrophotometer using quartz cells with a path length of 1 cm and a thermostatically controlled cell holder. The oligonucleotides were purified by HPLC on a Waters HPLC system consisting of a Waters 262 pump, a Waters 2407 UV detector, and a Waters 600S controller with a MonoHR 5/50 GL column. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. For FAAS analyses, DNA was precipitated with ethanol and dissolved in HCl (0.1 M). DPP was performed with an EG&G Princeton Applied Research Model 384B51sized filter paper and 1 mL of 1 M Tris-HCl (pH 7.4), MgCl2 (10 mM), and NaCl (150 mM). It was also verified, as described previously,[24,25] that the melting transitions of both the platinated and unmodified duplexes were fully reversible.
their most recent ideas in the field of anticancer metallodrugs with several European colleagues.


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DNA Duplexes Modified by Analogues of [PtCl(dien)]+


781