Temporal behavior of DNA thermal stability in the presence of platinum compounds. Role of monofunctional and bifunctional adducts

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Dedicated to the memory of Prof. Volodya I. Vardanyan
ABSTRACT
Penetrating into cell nuclei, antitumor drug cisplatin sequentially forms various intermediate and final adducts destroying local DNA structure. The demonstrated disappearance of the fine structure of melting curve of long DNAs along with a strong decrease in melting enthalpy conforms to the structural impact. However, the negative thermal effect ($\delta T_m$) caused by cisplatin is relatively small if neutral medium is used in melting experiments. Cisplatin's inactive analogs transplatin and diethylenetriaminechloroplatinum {Pt[(dien)Cl]Cl} also distort DNA structure but their thermal effect is even positive. We have found that the use of alkaline medium in melting experiments strengthens the negative thermal effect for cisplatin. For transplatin and Pt[(dien)Cl]Cl, the thermal effect becomes negative that makes it qualitatively consistent with structural distortions. Those changes are explained by elimination of nonspecific electrostatic stabilization of DNA under platination. Additionally, alkaline medium fixes intermediate states of DNA platination and makes them stable against heating. These results allowed us to monitor $\delta T_m$ under binding of platinum compounds to DNA and their further transformation. The kinetic and thermal characteristics of monofunctional and bifunctional adducts were evaluated. It has been demonstrated that monofunctional adducts of cisplatin, transplatin and Pt[(dien)Cl]Cl produce approximately the same thermal destabilization. Cisplatin intrastrand crosslinks cause a two-fold stronger thermal destabilization than its monofunctional adducts. The value of $\delta T_m$ for cisplatin’s final adducts is ten times larger than for transplatin. This difference mainly comes from the much stronger thermal destabilizing power of cisplatin’s intrastrand crosslinks, which are responsible for antitumor activity of this compound.

**Keywords:** DNA-complexes with platinum compounds; differential scanning calorimetry; optical melting studies; kinetics of DNA platination
1. Introduction

Cisplatin (cis-Pt(NH$_3$)$_2$Cl$_2$, Fig. 1A) is a widely used drug for the treatment of human cancers. Its biological activity is exerted by strong coordination binding to DNA [1-9]. However, cisplatin is highly cytotoxic and it has a number of side effects that limit its application [4,5]. Understanding the DNA binding mechanisms and anticancer properties of cisplatin and other platinum compounds may facilitate creation of new more effective drugs. Studies of cisplatin’s mechanisms often employ the very similar but inactive transplatin (trans-Pt(NH$_3$)$_2$Cl$_2$, Fig. 1B). In addition to transplatin, inactive diethylenetriaminechloroplatinum {[Pt(dien)Cl]Cl} is also employed in structure-activity studies (Fig. 1C).

Cisplatin include two amine non-leaving ligands and two labile chloride leaving groups (Fig. 1A). The major final product of the DNA-cisplatin reaction is intrastrand crosslinks between adjacent purines (Fig. 1e). The intrastrand crosslinks are responsible for the antitumor activity of this compound [1-5]. Their formation is slow and hindered by chloride ions. Therefore DNA platination in vitro is usually carried out in NaClO$_4$. The ion ClO$_4^-$ does not stop platination in contrast to Cl$^-$. The half time ($t_{1/2}$) of the first chloride dissociation and substitution with a water molecule is ~2 h [6] (Fig. 1b). Hydrolysis of the monoaquated form of cisplatin in the presence of DNA is followed by the rapid formation of intermediate monofunctional adducts mainly with the N7 atom of guanine (Fig. 1c). The monofunctional adduct undergoes dissociation of the second chloride and its replacement by a water molecule with approximately the same $t_{1/2}$ [6] (Fig. 1d). This reaction is followed by formation of a second coordination bond with the N7 atom of an adjacent guanine (5'-GpG) or adenine (5'-ApG) in the same strand (Fig. 1e). These intrastrand crosslinks account for 90% of cisplatin-DNA modifications. Approximately 6% of cisplatin-DNA adducts are interstrand crosslinks [10-12]. They are formed between neighboring guanine residues located in opposite strands (d(GpC)⋅d(GpC)) [13-15] (Fig. 1f). The final fraction of monofunctional adducts is negligible.

At high concentrations, cisplatin can additionally form long-range intrahelical and interhelical crosslinks [10,16].

Transplatin is the clinically inactive stereoisomer of cisplatin. It also preferentially reacts with guanine residues. However, in contrast to cisplatin, transplatin monofunctional adducts account for a considerable fraction of final products [6,9,11]. The structure of transplatin intrastrand crosslinks is GNG where N is a nucleotide of any type [9,17,18]. Closure of the transplatin monofunctional adduct to a bifunctional one is slower than for cisplatin, and more interstrand crosslinks occur [6,18,19-22]. The latter crosslinks are formed between complementary G and C residues of the same base pair and account for 12% of total transplatin-DNA modifications [11]. The exact ratio of final monofunctional adducts and intrastrand crosslinks is uncertain. Some studies suggest that transplatin forms only monofunctional adducts besides interstrand crosslinks [19, 20]. Other authors infer that intrastrand crosslinks are the main final product in double-stranded DNA [6,9,18]. The averaged data of various studies give the following fractions of adducts formed by transplatin: 35% of monofunctional adducts, 12% of interstrand and 53% of intrastrand crosslinks [6,9,11,23]. A review of this issue can be found in work [24].

[Pt(dien)Cl]Cl (Fig. 1C) forms only monofunctional adducts that makes it convenient for study of their properties.

Intermediate and final adducts formed by cisplatin, transplatin and [Pt(dien)Cl]Cl strongly distort the double helix [21,25-31]. These local distortions decrease the thermal stability of oligonucleotide duplexes ($\delta T_m<0$) [23,32-40].

In the case of long DNAs, there has been no clear correspondence between the distortion of DNA structure induced by platinum compounds and the decrease in thermal stability when measured in a medium with pH ~7 [23,35,41-52]. Only cisplatin caused thermal destabilization at any reasonable cation concentration demonstrating qualitative accordance with strong structural distortions [35,41-48]. However, the value of destabilization is small. Cisplatin's inactive analogs
transplatin and Pt[(dien)Cl]Cl also distort DNA structure but their thermal effect is even positive \([\delta T_m > 0]\).

In contrast to RNA, DNA is chemically stable in alkaline medium [53]. Therefore alkaline medium is used in DNA studies, purification and isolation methods. Here we have shown that the use of alkaline medium (pH~10.5) in melting experiments strongly strengthens the negative thermal effect \([\delta T_{m \text{pH 10.5}} > 0, \delta T_{m \text{pH 10.5}} < 0]\). For transplatin and [Pt(dien)Cl]Cl, the effect becomes negative \([\delta T_{m \text{pH 7}} \geq 0, \delta T_{m \text{pH 10.5}} < 0]\), i.e., at pH 10.5, the change in thermal stability appears to be related to the known structural distortions of DNA. At the same time, unmodified DNA conserves high thermal stability at pH 10.5 \([T_m \approx 65^\circ C \text{ at } [\text{Na}^+] \approx 0.1 \text{ M}]\) [49]. We have also demonstrated that intermediate states of DNA formed under platination at a given time of incubation \((t)\) become well fixed and stable against heating at pH 10.5 (this work and [54]). Those findings allowed us to measure the change in melting temperature as a function of time DNA is exposed to platination in neutral medium \([\delta T_m(t)]\). The dependence \([\delta T_m(t)]\) clearly shows the formation of intermediate monofunctional (Fig. 1c) and final bifunctional adducts of platination (Fig. 1e and 1f). It allows the simultaneous measurement of parameters characterizing their rate of formation and influence on DNA thermal stability. In all prior work, only the net effect of the final modifications was measured for long DNAs [23,35,41-52].

2. Experimental methods

Ultra pure calf thymus DNA that we prepared was used (protein<0.1%, RNA<0.1%, molecular mass ~30 MDa). The properties of this DNA have been previously described [55]. DNA at a concentration of 1.2 mg/mL was incubated in 10 mM NaClO₄ (pH~6) with cisplatin or transplatin of Sigma-Aldrich Corporation, or with Pt[(dien)Cl]Cl kindly provided by the Professor Natlie's laboratory. For measurements of melting temperature kinetics under platination, Pt/nucleotide molar ratio \((r)\) was 0.05. Cisplatin was added to DNA from a stock solution in distilled water (1 mg/mL) after several days of incubation at 25°C. This solution contains 35% of undissociated and 65% of monoaquated cisplatin [56]. The DNA-cisplatin mixture was incubated in 0.01 M NaClO₄ for 72 h at 37°C in the dark at pH~6. After 48-h incubation, cisplatin reacts with DNA quantitatively \((r_b=r)\) forming mainly bifunctional adducts [35,57,58]. At time intervals from 1 min to 72 h, aliquots of the reaction mixture were withdrawn. Platination in the aliquots was stopped by adjusting NaCl concentration to 0.1M. For optical melting studies, DNA solution was diluted to concentration 0.075mg/mL. Then the samples were frozen at -28°C [23,50,59]. Just before melting, DNA was thawed and diluted to 0.04 mg/mL. The optical melting was carried out in 0.1 M NaCl, 0.005M Na₂CO₃, 5⋅10⁻⁵M EDTA, pH 10.5 or pH 7. Depending on pH, the anion CO₃⁻ partially transforms into HCO₃⁻ and H₂CO₃. DNA melting was recorded by measuring the optical density at 260 nm as a function of temperature \((T)\) using a SF-26 spectrophotometer (LOMO, Russia).

High-resolution melting profiles of unmodified and platinated DNA were obtained using a model CSC 6300 NanoDSC differential scanning calorimeter (Calorimetry Sciences Corporation, USA) with a cell volume 0.3 mL. In the DSC experiments, we followed standard procedures [60].

The melting temperature was determined as a temperature corresponding to the half of the increase in light absorption (optical melting curves) or to the half of the excess heat absorption (calorimetry) caused by the helix-coil transition.

3. Results and discussion

3.1 Thermal stability of short and long platinated DNAs in neutral medium

Much progress has been made in understanding the mechanisms of antitumor effect of cisplatin [1-9]. Clinically ineffective trans isomer of cisplatin is used for comparison in studies of
biochemical, thermodynamic and structural properties of platinum antitumor compounds to reveal mechanisms underlying cisplatin's activity. Both compounds form monofunctional adducts, intrastrand crosslinks and interstrand crosslinks. [Pt(dien)Cl]Cl forms only monofunctional adducts and is useful for investigation of the properties of this adduct. Thermodynamic studies of these three compounds were carried out for short oligonucleotide duplexes of 10-20 bp. Monofunctional adducts [32,33,61] and intrastrand crosslinks [36-38] destabilize the duplexes at any cation concentration ($\delta T_m<0$). Interstrand crosslinks formed by cisplatin and transplatin increase oligonucleotide duplex melting temperature ($\delta T_m>0$) [39,40] because they strongly decrease the entropy of the melted state prohibiting strand separation [62,63]. Subtracting the nonspecific stabilization term caused by interstrand crosslinking, one obtains a negative $\delta T_m$ value that corresponds to the strong local distortions at sites of interstrand crosslinking with cisplatin or transplatin [39,40].

As follows from Fig. 2A, final adducts formed by cisplatin and transplatin in long DNA strongly decrease the melting enthalpy in neutral medium that is in agreement with distortions they cause in the double helix. The decrease in the enthalpy after platination by 0.9 and 1.5 kcal per mole of base pairs at $r_{f}=0.02$ means that every modification on an average decreases the enthalpy of helix-coil transition by 22.5 and 37.5 kilocalories per mole of modifications for cisplatin and transplatin, respectively. Earlier, the enthalpy decrease, slightly smaller in value, was found for oligonucleotide duplexes bound with cisplatin [36-40].

For unmodified DNA, the fine structure of calorimetric melting curve is well seen in Fig. 2A. Both cisplatin and transplatin destroys it (see also [64]). Earlier, a similar destruction was found for plasmid DNA [65]. The fine structure observed in differential melting curves of higher organisms' DNA arises from melting of long (up to several megabases) periodical sequences of satellite DNA mainly located in chromosome centromeres. For different organisms, the length of periods varies from one base pair to several thousand. The satellite sequences give rise to a group of narrow melting bands that are different for various biological species. In calf thymus DNA, they account for more than 15% of total genome and give rise to four narrow melting bands, which form the fine structure. The satellite bands are well seen against the background of the smooth broad main band. That band corresponds to melting of all other sequences much more heterogeneous than satellite DNA.

Originating new types of DNA units, platination strongly increases general DNA heterogeneity. A relative increase in heterogeneity is higher for satellite DNA that looses periodicity of its primary structure after introduction of randomly distributed platinated sites. Therefore satellite bands decrease the heights and increase the widths much stronger than the main band. As a result, the fine structure disappears.

In contrast to elimination of fine structure and a decrease in enthalpy of the helix-coil transition, a change in thermal stability ($\delta T_m$) of long DNAs does not conform to structural distortions caused by the platinum compounds. Of the three compounds, only cisplatin decreased melting temperature of long DNAs [35,41-48]. For [Pt(dien)Cl]Cl and transplatin, the value of $\delta T_m$ is positive at low [Na$^+$] and tends to zero at [Na$^+$]=0.1-0.2 M. Even more surprising is the observation that [Pt(dien)Cl]Cl and transplatin complexes with long DNAs demonstrate the same melting behavior in neutral medium in spite of different final adducts [23,35]. The absence or low value of a decrease in thermal stability under platination of long DNAs can be caused by two effects that compensate thermal destabilization originating from structural distortions [35,49,66]. First, all modifications decrease DNA charge density, and therefore they stabilize the double helix [35,49,67]. The fact that maximal destabilization for cisplatin and minimal stabilization for transplatin and [Pt(dien)Cl]Cl are reached at high Na$^+$ concentration (0.1-0.2 M) supports this viewpoint. Electrostatic interactions for long DNA are stronger than for short oligonucleotide duplexes. Therefore, the thermal stabilization is also stronger for long molecules. Second, as shown from our studies [49,66], interstrand crosslinking can increase thermal stability of long DNAs under some conditions. As a result, the total thermal destabilizing effect of cisplatin is not large in neutral medium in spite of the strong structural distortions it causes in DNA (see [49,66] and Fig. 2B).
3.2 The influence of platinum compounds on thermal stability of long DNA in alkaline medium

To study thermal impact of final adducts (Fig. 1e and 1f), a 48-h incubation of DNA and cisplatin was carried out in 0.01M NaClO₄ at 37°C and pH~6 for various cisplatin / nucleotide molar ratios \( r \). The influence of \( r \) on melting temperature \( \Delta T_m \) was measured in 0.1M NaCl, 0.005M Na₂CO₃, 0.001M NaClO₄, 5⋅10⁻⁵M EDTA, pH 10.5 and in the same solution at pH 7. Although the melting temperature of unplatinated DNA is ~20°C lower in the alkaline medium than in neutral solution (a decrease from 85 to 65°C), the high hyperchromicity and cooperative melting transitions demonstrate that the DNA remains initially a double helix.

A negative shift in melting temperature reached after 48-h incubation at pH 7 is weak for cisplatin (Fig. 2B and [35]). Alkaline medium strengthened it (Fig. 2B). As shown below, if alkaline medium is used for melting studies, thermal effect for transplatin and [Pt(dien)Cl]Cl changes its sign and becomes negative as consistent with their structural distortions.

In kinetic studies, platination was stopped by addition of NaCl. In concentration 0.1 M, chloride ions stop platination in neutral medium at moderate and low temperatures \[23,49,52,59,68\]. However, we have shown that intermediate states of DNA arisen under platination are not stable at the high temperatures of melting experiments, and DNA platination continues to develop in 0.1 M NaCl solution at neutral pH \[54\]. At the same time, the use of alkaline medium in melting studies stops the development of DNA platination and fixes the states formed under platination at a given time of incubation \( t \). To prove this fact, cisplatin from a stock solution where 65% of the cisplatin is in the monoaquated form was added at \( r = 0.05 \) to a DNA solution containing 0.1M NaCl, pH 7. Then measurements of DNA melting curves were conducted in alkaline solution (0.1M NaCl, 0.005M Na₂CO₃, 0.001M NaClO₄, 5⋅10⁻⁵M EDTA, pH 10.5). The difference in melting temperature with and without cisplatin was less than 0.5°C (data not shown). Taking into account that a 48 h incubation of DNA with cisplatin in 0.01 M NaClO₄ at 37°C and pH 6 causes a decrease in melting temperature of 12.2±1.5°C (Fig. 2B), one can conclude that additional platination of DNA is minimal during incubation in 0.1 M NaCl at neutral pH at 37°C followed by melting in alkaline medium. Thus alkaline medium not only increases the impact of cisplatin on thermal stability, but makes intermediate monofunctional products stable against high temperatures of melting experiments and, in general, fixes intermediate states of DNA arisen to a given time of incubation \( t \).

If melting is carried out at pH 7, the curve \( \Delta T_m(t) \) is weakly dependent on cisplatin binding and further transformation (Fig. 3A). Alkaline medium creates a specific character of dependences \( \Delta T_m(t) \) and provides conditions for thermal kinetic studies. It is seen that the DNA melting temperature decreases during 7 h of incubation with cisplatin in 0.01 M NaClO₄ at 37°C and pH 6 causes a decrease in melting temperature of 12.2±1.5°C (Fig. 2B), one can conclude that additional platination of DNA is minimal during incubation in 0.1 M NaCl at neutral pH at 37°C followed by melting in alkaline medium. Thus alkaline medium not only increases the impact of cisplatin on thermal stability, but makes intermediate monofunctional products stable against high temperatures of melting experiments and, in general, fixes intermediate states of DNA arisen to a given time of incubation \( t \).

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The two-step character of the kinetic curve is well seen in Fig. 3. The quick step is characterized with \( t_{1/2}=1.2\text{min} \) and corresponds to formation of monofunctional adducts. For the slow step corresponding mainly to bifunctional adducts, \( t_{1/2}=2\text{h} \). A scheme shown in Figure 1D explains the second step but cannot clarify a quick origin of the first one. As follows from Fig. 1b, cisplatin activation is caused by the replacement of a chloride with a water molecule, and \( t_{1/2} \) for the dissociation of the first chloride to form the monoaquated species of cisplatin is about 2 h \[3,6\]. Therefore activation is a slow process followed by quick formation of a monofunctional adduct. For such system, a large fraction of monofunctional adducts cannot be formed because they transform into bifunctional ones with \( t_{1/2} \approx 2\text{h} \). However, as observed in Fig. 3, monofunctional adducts are formed quickly and their fraction is sufficient to decrease the thermal stability.

This rapid formation of monofunctional adducts and a corresponding change in thermal stability is caused by the preliminary incubation of cisplatin in distilled water for several days at 25°C and
pH 5.6. This incubation changes the scheme of platination shown in Fig. 1D. According to the analysis of Yotsuyanagi et al. [56], the stock solution of cisplatin employed contained approximately 35% of undissociated and 65% of monoaquated cisplatin. The equilibrium state is reached in 25 h [69]. Thus, for 65% of cisplatin, the transformation begins from the stage shown in Fig. 1b. The monofunctional adducts quickly arise from monoaquated cisplatin ($t_{1/2} \approx 1$ to 6 min [6] and references therein). Those quickly formed monofunctional adducts slowly transform into bifunctional ones:

$$\text{monoaquated cisplatin} \rightarrow \text{monoadducts} \rightarrow \text{biadducts} \quad (1)$$

It means that during the first few minutes of incubation a large fraction of monofunctional adducts is formed (about 65% of total cisplatin introduced in solution). Thus at $t=8$ min, there are mainly monofunctional adducts (~65%) and unbound undissociated cisplatin (~35%). The thermal impact of these 65% of monofunctional adducts can be easily measured because the fraction of bifunctional adducts is low at $t=8$ min.

Slow parallel formation of monofunctional adducts from 35% of total cisplatin added to DNA in undissociated form, and its further transformation into monofunctional and bifunctional ones occurs by Eq. 2:

$$\text{cisplatin} \rightarrow \text{monoaquated cisplatin} \rightarrow \text{monoadducts} \rightarrow \text{biadducts} \quad (2)$$

Thus, the rapidly formed monofunctional adducts (Eq. 1) give rise to the first step of the curve $\delta T_m(t)$. All slow processes, i.e., formation of monofunctional adducts from undissociated cisplatin (Eq. 2) and bifunctional adducts from monofunctional ones (Eqs. 1 and 2) gives rise to the second step (Fig. 3). To better visualize the slow and quick steps the dependence $\theta(t) = \delta T_m(t)/\delta T_{max}$ was differentiated (Fig. 3B, curve 1). The constituent contributions to curve 1 are more readily visualized in the differential curve 2. The initial time course of the larger constituent 2b was determined by extrapolation. The less well resolved constituent 2a was calculated as the difference between curve 2 and constituent 2b. Using curves 2a and 2b, constituents 1a and 1b of initial curve 1 were calculated (Fig. 4). Constituent 1a is characterized with $t_{1/2a}=1.2$ min (Fig. 4). The height of this constituent $h_{1a}=0.29$ corresponds to the thermal effect of monofunctional adducts that are quickly formed by monoaquated cisplatin in the stock solution where its fraction is 65% [56]. The maximal fraction of these monofunctional adducts is reached at $t \approx 8$ min (Fig. 4). Constituent 1b in Fig. 4 corresponds to the relatively slow process of binding and transformation given by the last stage of Eq. 1 and all transformations in Eq. 2. It is characterized by a $t_{1/2b}=120$ min and a height $h_{1b}=0.71$. Formation of a large fraction of monofunctional adducts sufficient to measure their impact on DNA thermal stability before formation of bifunctional ones (Eq. 1) is a result of preincubation of cisplatin stock solution in distilled water. If there is no preincubation or 0.01 M NaCl is added to the stock solution of cisplatin or to the medium used for incubation of cisplatin and DNA, then the fraction of monofunctional adducts sufficient for the measurement of their thermal impact is not formed.

The time dependence of DNA thermal stability was also measured for transplatin, a very similar but inactive analog of cisplatin, and for $[\text{Pt(dien)}\text{Cl}]\text{Cl}$ that forms only monofunctional adducts. Cisplatin decreases DNA thermal stability monotonously (Fig. 3). In contrast, transplatin produces a maximal destabilization in three hours ($\delta T_m(t=3h)=-5^\circ C$) and then, at longer times, the DNA’s melting temperature increases (Fig. 5). However, it does not reach the value corresponding to the unmodified DNA molecule. If DNA melting curves are registered in a neutral medium (pH~7), the curve of $\delta T_m$ vs. incubation time $t$ does not suggest that transplatin binds with DNA (Fig. 5, curve "trans, pH 7"). Since the maximal $|\delta T_m(t)|$ for transplatin is three times lower than for cisplatin at $r=0.05$, we have repeated the measurements for transplatin at $r = 0.15$ to equalize the maximal destabilization with the value obtained for cisplatin at $r = 0.05$. The kinetic curve for $r = 0.15$
conserves the shape with an increase in effective values. [Pt(dien)Cl]Cl monotonically decreases the thermal stability of DNA similar to cisplatin, and $\delta T_{\text{max}} = -8^\circ C$. However, the saturation is reached more rapidly ($t=2\ h$) and $t_{1/2}=20\ \text{min}$.

3.3 The mechanism of a stronger decrease in thermal stability of platinated DNA in alkaline medium

It is known that positively charged polyions such as histones, polylysine and protamines strongly increase the DNA melting temperature ($\delta T_m>0$). This thermal stabilization weakens with Na$^+$ concentration [70,71]. The melting temperatures of bound and free DNA becomes equal ($\delta T_m=0$) at sodium ions concentration 0.5 M for protamines and 0.65 M for polylysine. In a similar way, platinum compounds increase melting temperature. The charge density decreases around disordered sites of platination that gives compensating thermal stabilization. This thermal stabilization hides the destructive structural effect on DNA thermal stability, especially at low [Na$^+$] [35,49]. For [Pt(dien)Cl]Cl, which forms only monofunctional adducts, and for transplatin, almost full reciprocal compensation of electrostatic stabilization and structural destabilization occurs at [Na$^+$] = 0.1-0.2 M [23,35,50]. For cisplatin, this critical Na$^+$ concentration is much lower. Therefore destabilization takes place at any reasonable [Na$^+$], but it is relatively weak even at [Na$^+$] = 0.1-0.2 M ([23,35] and Fig. 2B).

We have found increased thermal destabilization of DNA with cisplatin if melting experiments are carried out in alkaline medium (pH~10.5) (Fig. 2B and [49]). In these conditions, both [Pt(dien)Cl]Cl and transplatin also cause DNA destabilization (Fig. 5). Thus, alkaline medium makes DNA thermal destabilization common to all considered platinum compounds. On the other hand, DNA is chemically stable at pH 10.5 [53]. That allows the use of alkaline medium in various DNA studies and in methods of isolation of plasmid DNA. Such stability is not inherent to RNA in alkaline medium as well as for DNA in acidic medium [53].

The enhancement of thermal destabilization in alkaline medium can be explained by the peculiarities of DNA deprotonation. DNA deprotonation causes a destabilizing effect at pH>10, i.e., when pH is higher than pK's of nitrogen bases (see [53,72] for detail). In alkaline medium, thermal destabilization takes place for both unmodified and platinated DNA because the majority protons able to dissociate from DNA in alkaline medium are included in the formation hydrogen bonds that generate the double helix [53,72]. Binding of platinum compounds decreases DNA charge density in helical regions adjacent to destroyed sites of platination. In alkaline medium, this facilitates additional proton dissociation from those regions and the corresponding additional decrease in thermal stability. Thus, the stabilizing electrostatic effect of platination is compensated in alkaline medium by destabilizing influence of stronger deprotonation. As a result, the absolute value of a negative change in melting temperature ($\delta T_m$) caused by platination strongly increases relative to the neutral medium that was used in previous thermodynamic and thermal studies (Fig. 2B).

Eliminating electrostatic interactions, alkaline medium makes the influence of platinum compounds on long DNA similar to their impact on nucleotide duplexes.

As we have shown earlier, an increase in Na$^-$ concentration strengthens the negative shift of melting temperature as medium is changed from neutral to alkaline because of a strong decrease in pK values of nucleotide deprotonation with [Na$^-$] [53,72]. That fact also supports the proposed electrostatic mechanism of additional destabilization of platinated DNA in alkaline medium.

3.4 The effect of interstrand crosslinks on DNA thermal stability

Interstrand crosslinks influence DNA thermodynamic properties in two ways. Besides a local change in DNA structure and thermal stability that is generated by all types of chemical modifications, interstrand crosslinks increase the thermal stability of short DNAs prohibiting strand dissociation [39,40,62,63]. Additional stabilization of long DNAs is caused by formation of additional loops in melted regions [73-78]. The fraction of interstrand crosslinks accounts for 6%
and 12% for cisplatin and transplatin, respectively [10-12]. At the \( r = 0.05 \) value used in this study, their per nucleotide number is 0.003 and 0.006. As follows from our recent calculations [49,66,77], interstrand crosslinks of the relative concentration 0.003 increase melting temperature of long DNA by 1.3°C if they do not cause distortions at sites of their location and by 1.6°C in the case of strong local stabilization. This corresponds to approximately 10% of the total change in \( \Delta T_m \) caused by all final modifications of cisplatin in alkaline medium (~12°C, Fig. 3A). Thus, even the strongest possible impact of cisplatin interstrand crosslinks on thermal stability is weak. However, cisplatin and transplatin interstrand crosslinks cause local structural destabilization besides stabilizing crosslinking effect [39,40]. From the data on melting of oligonucleotide duplexes [39], one can obtain that local distortions formed by cisplatin and transplatin interstrand crosslinks give rise to the following change in the free energy (kcal/mol duplexes) of the helix-coil transition: 

\[
\Delta G = -18 + 0.037 \cdot T_K \quad \text{and} \quad \Delta G = -2 - 0.015 \cdot T_K,
\]

respectively. Our calculations [49,66,78] demonstrate that, in long DNA, such interstrand crosslinks cause a very small change in thermal stability (less than 0.3°C). These estimations are valid for alkaline medium where a stabilizing electrostatic effect is eliminated that makes the situation similar to the case of oligonucleotide duplexes. Therefore the thermal effect of interstrand crosslinking will be not taken into account in further consideration. However, stabilizing effect of crosslinking can be much stronger in neutral medium where electrostatic stabilization is not eliminated.

3.5 Cisplatin

Cisplatin's kinetic curve \( \Delta T_m(t) \) reflects cisplatin transformations shown in Fig. 1D. During the first minutes of incubation, a decrease in melting temperature is caused by monofunctional adducts formed by monoaquated cisplatin arisen during preincubation of cisplatin solution. Appropriate constituent 1a is characterized with \( t_{1/2a}=1.2 \) min (Fig. 4). The height \( h_{1a}=0.29 \) (\( \Delta T_m=-3.5°C \)) corresponds to its fraction of 65% [56]. For fully monoaquated cisplatin, the height of the first constituent would be equal to 0.45 (\( h_{1\text{max}}=h_{1a}/0.65=0.45; \Delta T_m=-5.5°C, r=0.05 \)). It is the maximal relative change in melting temperature that can be caused by monofunctional adducts when all cisplatin forms this modification only.

Constituent 1b in Fig. 4 corresponds to slow formation of monofunctional adducts from nonaquated cisplatin and transformations of monofunctional adducts arisen from primarily nonaquated and monoaquated cisplatin into bifunctional adducts. Constituent 1b is characterized with \( t_{1/2b}=120 \) min, and its height \( h_{1b}=0.71 \). Both \( t_{1/2a} \) and \( t_{1/2b} \) values are in agreement with previous studies [6]. Computer modeling demonstrates that formation of monofunctional adducts from undissociated cisplatin weakly change constituent 1b (not shown). Therefore constituent 1b mainly reflects formation of final bifunctional adducts. For cisplatin, almost all monofunctional adducts transform into bifunctional ones. The final products formed after 48 h of incubation are mainly intrastrand and interstrand crosslinks. As follows from Fig. 4, their relative thermal effect is always equal to unity (\( h_{1a}+h_{1b}=h_1=1 \) (\( \Delta T_m=-12.2°C, r=0.05 \))) independently of content of cisplatin species in stock solution. As follows from these data, the destabilizing thermal effect of bifunctional adducts (\( h_{1a}+h_{1b}=h_1=1 \)) is 2.2-fold stronger than that of monofunctional ones (\( h_{1\text{max}}=0.45 \)).

3.6 Transplatin and its comparison with cisplatin

Transplatin is used as an inactive reference in various investigations to reveal the mechanism of anticancer activity of cisplatin and other platinum compounds. The averaged data of various studies give the following fractions of adducts formed by transplatin: 35% of monofunctional adducts, 12% of interstrand and 53% of intrastrand crosslinks [6,9,11,23,79-81]. In contrast to cisplatin (Fig. 3A), the dependence \( \Delta T_m(t) \) is not monotonic for transplatin (Fig. 5). As for cisplatin, primary decrease in melting temperature is caused by formation of monofunctional adducts. However, they are accumulated in larger amount because the formation of bifunctional
adducts for transplatin is much slower. Therefore, their fraction gains larger values and the impact on intermediate thermal stability (-5°C) is stronger than for cisplatin (-3.5°C). However, approximately the same decrease can be reached if all cisplatin transforms into monofunctional adducts only (δT_m=5.5°C). [Pt(dien)Cl]Cl which forms only monofunctional adducts gives a similar final destabilization (δT_m=8°C, Fig. 5). Transplatin interstrand crosslinks account for 12% of all modifications [11], but, as explained above, they do not influence the thermal stability in long DNA because of structural distortions at sites of their location.

If δT_m=−5°C at t=3h corresponds to destabilization caused by almost all transplatin monofunctional adducts, then the final 35% that do not transform into bifunctional ones give δT_m=−1.7°C. Our experiment gives approximately the same final value of δT_m=−1.5°C at t=72 h (Fig. 5). It suggests the impact of interstrand and intrastrand crosslinks on thermal stability is much lower relative to monofunctional adducts, and an increase in δT_m(t) for t>3h is mainly caused by reduction of the number of destabilizing monofunctional adducts to 35%. Thus, intrastrand crosslinks formed by transplatin give weak thermal effect for long DNA in contrast to strong destabilization caused by cisplatin intrastrand crosslinks.

4. Conclusion

Penetrating into cell nuclei, antitumor drug cisplatin interacts with DNA and sequentially forms various intermediate monofunctional and final bifunctional adducts. We found conditions to follow impact of these chemical modifications on DNA thermal stability (δT_m) under their transformation. It was shown that, in alkaline medium, intermediate states of DNA formed under platination at a given time of incubation became well fixed and stable against high temperature of melting experiments. Additionally, because of elimination of nonspecific electrostatic stabilization, the negative thermal effect strongly strengthened for cisplatin. For inactive transplatin and [Pt(dien)Cl]Cl, the thermal effect became negative (δT_m<0) in contrast to neutral medium where δT_m≥0. Thus the thermal effect for long DNA became consistent with the structural distortions of the double helix, a decrease in the enthalpy of the helix-coil transition and disappearance of the fine structure of melting curves. The proposed approach allowed us to refine t_1/2=1.2 min for transformation of monoaquated cisplatin into monofunctional adducts and confirm t_1/2~2 h of formation of bifunctional adducts from monofunctional. More recent antitumor platinum compounds carboplatin and oxaliplatin demonstrate much slower kinetics [82-84]. In the alkaline conditions, monofunctional adducts formed by cisplatin, transplatin and [Pt(dien)Cl]Cl cause thermal destabilization that is not too different in value (-5 to -8°C, r=0.05). Cisplatin intrastrand crosslinks give rise to a larger decrease in melting temperature than its monofunctional adducts. In contrast, transplatin intrastrand crosslinks cause low destabilizing thermal effect relative to its monofunctional adducts. A ten time difference in DNA thermal destabilization caused by cisplatin and transplatin final adducts mainly comes from much stronger destabilizing power of cisplatin intrastrand crosslinks that are responsible for the antitumor activity of this compound.

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References

Figure Legends

**Fig. 1.** A) Cisplatin. B) Transplatin. C) [Pt(dien)Cl]Cl. D) DNA chemical modification with cisplatin: a) Cisplatin after quick solution in water retains both chlorides; b) Slow dissociation of the first chloride and its substitution with H2O, t1/2~2h; c) Quick formation of a monofunctional adduct with guanine; d) Slow dissociation of the second chloride and its substitution with H2O, t1/2~2h; e) Quick formation of an intrastrand crosslink between two neighboring guanines or between guanine and adenine of the same DNA strand; f) Quick formation of an interstrand crosslink between two neighboring guanines of different DNA strands.

**Fig. 2.** A) The high resolution calorimetric melting profile for calf thymus DNA before and after its chemical modification with cisplatin and transplatin at per nucleotide concentration rb=0.02. Melting was carried out at 0.21 M Na⁺ and pH 7. Corresponding enthalpy values are 9.2, 8.3, and 7.7 kcal/(mol bp). B) The dependence of the shift of melting temperature (δTm) caused by cisplatin on rb for melting carried out in neutral (pH 7) and alkaline media (pH 10.5) at 0.1 M Na⁺. Complexes were formed by a 48-h incubation of cisplatin and DNA in 0.01M NaClO₄ at 37°C and pH~6.

**Fig. 3.** A) The time dependence of a change in DNA melting temperature caused by cisplatin binding in 0.01 M NaClO₄ at 37°C (pH 6) and r=0.05. DNA melting curves were measured at pH 10.5 or pH 7. t1/2 for a decrease in melting temperature is equal to 70 min. B) The time dependence of a relative change in DNA melting temperature caused by cisplatin θ(t)=δTm(t)/δTmax (transformation of Figure 3A) (1); the first derivative of curve 1 with respect to lg(t) (2), and its constituents 2a and 2b.

**Fig. 4.** The time dependence of a relative change in DNA melting temperature caused by binding of cisplatin (1), and constituents 1a and 1b. t1/2a=1.2 min, t1/2b=120 min.

**Fig. 5.** A change in DNA melting temperature caused by binding of transplatin at r=0.05, 0.15 and [Pt(dien)Cl]Cl at r=0.05. DNA melting curves were measured at pH 10.5 (solid symbols) or pH 7 (open circles).
Fig. 1. A) Cisplatin. B) Transplatin. C) [Pt(dien)Cl]. D) DNA chemical modification with cisplatin: a) Cisplatin after quick solution in water retains both chlorides; b) Slow dissociation of the first chloride and its substitution with H₂O, \( t_{1/2} \sim 2h \); c) Quick formation of a monofunctional adduct with guanine; d) Slow dissociation of the second chloride and its substitution with H₂O, \( t_{1/2} \sim 2h \); e) Quick formation of an intrastrand crosslink between two neighboring guanines or between guanine and adenine of the same DNA strand; f) Quick formation of an interstrand crosslink between two neighboring guanines of different DNA strands.
Fig. 2. A) The high resolution calorimetric melting profile for calf thymus DNA before and after its chemical modification with cisplatin and transplatin at per nucleotide concentration $r_b=0.02$. Melting was carried out at 0.21 M Na$^+$ and pH 7. Corresponding enthalpy values are 9.2, 8.3, and 7.7 kcal/(mol bp). B) The dependence of the shift of melting temperature ($\delta T_m$) caused by cisplatin on $r_b$ for melting carried out in neutral (pH 7) and alkaline media (pH 10.5) at 0.1 M Na$^+$. Complexes were formed by a 48-h incubation of cisplatin and DNA in 0.01M NaClO$_4$ at 37°C and pH~6.
Fig. 3. A) The time dependence of a change in DNA melting temperature caused by cisplatin binding in 0.01 M NaClO₄ at 37°C (pH 6) and $r=0.05$. DNA melting curves were measured at pH 10.5 or pH 7. $t_{1/2}$ for a decrease in melting temperature is equal to 70 min. B) The time dependence of a relative change in DNA melting temperature caused by cisplatin $\theta(t)=\delta T_m(t)/\delta T_{max}$ (transformation of Figure 3A) (1); the first derivative of curve 1 with respect to lg(t) (2), and its constituents 2a and 2b.
Fig. 4. The time dependence of a relative change in DNA melting temperature caused by binding of cisplatin (1), and constituents 1a and 1b. $t_{1/2a}=1.2$ min, $t_{1/2b}=120$ min.
Fig. 5. A change in DNA melting temperature caused by binding of transplatin at \( r = 0.05 \), 0.15 and [Pt(dien)Cl]Cl at \( r = 0.05 \). DNA melting curves were measured at pH 10.5 (solid symbols) or pH 7 (open circles).
Highlights

- Alkaline medium is used in melting studies of DNA complexes with platinum compounds.
- At pH 10.5, the thermal DNA destabilization under platination is much stronger.
- The mechanism relies on promotion of DNA deprotonation under platination.
- Alkaline medium stabilizes intermediate states of DNA platination against heating.
- Thermal impact of monofunctional and bifunctional platinum adducts was evaluated.