Mechanism of the formation of DNA–protein cross-links by antitumor cisplatin

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Received October 5, 2006; Revised January 2, 2007; Accepted January 5, 2007

ABSTRACT

DNA–protein cross-links are formed by various DNA-damaging agents including antitumor platinum drugs. The natures of these ternary DNA–Pt–protein complexes (DPCLs) can be inferred, yet much remains to be learned about their structures and mechanisms of formation. We investigated the origin of these DPCLs and their cellular processing on molecular level using gel electrophoresis shift assay. We show that in cell-free media cisplatin [ cis-diamminedichloroplatinum(II) ] forms DPCLs more effectively than ineffective transplatin [ trans-diamminedichloroplatinum(II) ]. Mechanisms of transformation of individual types of plain DNA adducts of the platinum complexes into the DPCLs in the presence of several DNA-binding proteins have been also investigated. The DPCLs are formed by the transformation of DNA monofunctional and intrastrand cross-links of cisplatin. In contrast, interstrand cross-links of cisplatin and monofunctional adducts of transplatin are stable in presence of the proteins. The DPCLs formed by cisplatin inhibit DNA polymerization or removal of these ternary lesions from DNA by nucleotide excision repair system more effectively than plain DNA intrastrand or monofunctional adducts. Thus, the bulky DNA–protein cross-links formed by cisplatin represent a more distinct and persisting structural motif recognized by the components of downstream cellular systems processing DNA damage considerably differently than the plain DNA adducts of this metallodrug.

INTRODUCTION

cis-Diamminedichloroplatinum(II) (cisplatin) (Figure 1A) has been widely used in chemotherapy for almost 30 years. Hence, mechanisms underlying biological effects of this purely inorganic, simple, but outstanding compound have been intensively examined. In spite of this intensive research lasting more than three decades, many important details of the mechanism of anticancer effects of cisplatin have not been so far clarified. It is generally accepted that the major pharmacological target of cisplatin and other platinum anticancer compounds is DNA (1) and that the cytotoxicity of platinum compounds is thought to be determined primarily by their DNA adducts (2). Numerous studies show that cisplatin forms in DNA ~90% intrastrand cross-links (CLs) between neighboring purine bases (1,2-GG or 1,2-AG intrastrand CLs) and remaining lesions are intrastrand CLs between purine bases separated by a third base, interstrand CLs and monofunctional adducts (3) Structures, other physical properties of these adducts and their recognition by the components of downstream cellular systems processing DNA damage have been intensively studied (4–6).

Importantly, some studies indicate that cisplatin forms besides these DNA adducts also ternary DNA–platinum–protein CLs [see Wozniak and Walter (7) for review]. For instance, cisplatin has been reported to cross-link chromosomal proteins, including histones or cytokeratins, to DNA (8,9). Interestingly, the DPCLs (formed by antitumor trans-[PtCl2(E-iminoether)2]) inhibit DNA replication, DNA repair and are recognized by cellular components distinctly differently than plain Pt–DNA adducts.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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MATERIALS AND METHODS

Starting material

Cisplatin, transplatin and dimethyl sulfate (DMS) were from Sigma (Prague, Czech Republic). The stock solutions of platinum compounds were prepared at the concentration of $5 \times 10^{-4}$ M in 10 mM NaClO$_4$ and stored at 4°C in the dark. The synthetic oligodeoxyribonucleotides used in this work (Figure 1B) were purchased from VBC-genomics (Vienna, Austria). The purity of compounds was verified by high-pressure liquid chromatography (HPLC) or gel electrophoresis. T4 polynucleotide kinase and NdeI restriction endonuclease were purchased from New England Biolabs (Beverly, MA). The Klenow fragment from DNA polymerase I (exonuclease minus, mutated to remove the $3' \rightarrow 5'$ proofreading domain) (KF$^-$). NdeI and EcoRI restriction endonucleases were purchased from Takara (Japan). Histone H1 was from Roche diagnostics, GmbH (Mannheim, Germany). NF-xB protein (p50 homodimer) was kindly provided by Prof. Vašák (University of Zürich, Switzerland). Acrylamide, bis(acrylamide), dithiothreitol (DTT), urea and NaCN were from Merck KgaA (Darmstadt, Germany). Agarose and Metaphor agarose were from FMC BioProducts (Rockland, ME, USA). Sodium dodecyl sulphate (SDS) was from Serva (Heidelberg, Germany). Wizard$^\text{®}$ SV and PCR Clean-Up System used to extract and purify 213-base pairs (bp) DNA fragment (vide infra) was purchased from Promega. Nonidet P-30 was from Fluka (Prague, Czech Republic). $[^{32}\text{P}]$-ATP and [$^{25}\text{P}$]-dATP were from MP Biomedicals, LLC (Irvine, CA). Proteinase K was from Boehringer (Mannheim, Germany).

Isolation and purification of a 213-bp DNA fragment

A double-stranded DNA probe was prepared by digesting the pUC19 plasmid with NdeI and EcoRI. The resulting two fragments, 2473- and 213-bp long, were separated by agarose gel electrophoresis. T4 polynucleotide kinase and 5 U of KF$^-$ were added and incubated for additional 1 h. Unincorporated label was removed on column filled with sephadex G50 coarse, DNA was extracted with phenol/chloroform (1:1), ethanol precipitated and dissolved in 0.1 M NaClO$_4$. The fragment was then globally modified with cisplatin or transplatin for 24 h in 0.1 M NaClO$_4$ to an $r_b$ value of 0.02 ($r_b$ is defined as the amount of platinum atoms bound per one nucleotide in DNA).

Platination of oligonucleotides

Single-stranded oligodeoxyribonucleotide probes (40mer for the reaction with KF$^-$ and histone H1 and 21mer for reaction with NF-xB) were 5'-end labeled with T4 polynucleotide kinase and $[^{32}\text{P}]$-ATP and allowed to anneal with complementary strands in 0.1 M NaClO$_4$. This annealing procedure included a rapid heating of the mixture of the complementary oligonucleotides to 75°C followed by the incubation at 25°C for 2 h. Double-stranded oligonucleotides were then globally modified with cisplatin or transplatin for various time intervals in the range of 0–24 h. The final $r_b$ values at the end of these reactions were in the range of 0.01–0.05. The platination reaction was terminated by addition of sodium acetate...
so that its resulting concentration was 0.3 M followed by ethanol precipitation.

The 20-bp duplexes containing single, site-specific monofunctional, intrastrand or interstrand CLs were prepared as described in the previously published articles and also in the Supplementary Material

**Preparation of the protein samples**

The final composition of the storage buffers: KF\(^-\): 10 mM Tris pH 8, 0.5 mM EDTA, 100 μg/ml bovine serum albumin (BSA), 50% glycerol and 2 mM MgSO\(_4\); histone H1: 10 mM Tris pH 7.9, 20 mM NaCl, 0.1 mM PMSF for histone H1; NF-κB protein (p50 dimer): 25 mM Tris/HCl pH 8.0, 50 mM NaCl. The commercially available sample of KF\(^-\) was in the manufacturer’s storage buffer containing DTT; the manufacturer’s storage buffer was exchanged for that specified earlier using microcon concentrators.

**Formation of the ternary DNA–platinum–protein complexes**

Platinated DNA (213-bp fragment or oligodeoxyribonucleotide duplexes) at the concentration of 10 nM was incubated with the proteins (KF\(^-\), histone H1 or NF-κB) at the concentration of 100 nM overnight at room temperature in the appropriate buffer: 10 mM Tris pH 8, 10 mM EDTA, 0.1 mM BSA, 0.8% glycerol and 2 mM MgSO\(_4\) (KF\(^-\)); 10 mM Tris pH 7.9 and 20 mM NaCl (histone H1); 42 mM HEPES, 42 mM KCl, 1 mM MgCl\(_2\), 0.02 mM EDTA, 210 mM DDT, 2.5% glycerol and 2% Ficoll (NF-κB). The ability to form CLs by cisplatin or transplatin between oligonucleotide duplexes (20–40 bp) and proteins was assessed by 10% SDS/polyacrylamide (PAA) gel electrophoresis after mixing the samples with the loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and denaturing by heat at 90°C for 5 min. Gels were electrophoresed for 1–2 h at 140 V, dried and visualized by using the bio-imaging analyzer. The ability to form CLs by cisplatin between 213-bp DNA fragment and proteins was assessed by 10% SDS/polyacrylamide (PAA) gel electrophoresis after mixing the samples with the loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and denaturing by heat at 90°C for 5 min. Gels were electrophoresed for 2 h at 50 V, dried and visualized by bio-imaging analyzer.

**Transformation of single, site-specific adducts of cisplatin and transplatin into DNA–platinum–protein cross-links**

The series of single, site-specific adducts of cisplatin and transplatin were examined for their ability to isomerize and form ternary DPCLs; the 20-bp duplexes containing single, site-specific 1,2-GG intrastrand CL, interstrand CL of cisplatin or monofunctional adduct of cisplatin or transplatin were prepared as described earlier. The platinated duplexes at the concentration of 100 nM were incubated with KF\(^-\) (100 nM) at 25°C for various time intervals and the formation of the DNA–Pt–protein ternary complexes was assessed by 10% SDS–PAA gel electrophoresis as described earlier.

**Inhibition of DNA polymerization**

The 44mer templates unplatinated or containing single 1,2-GG intrastrand CL of cisplatin were prepared in the same way as described in the Supplementary material. The 44mer template cross-linked to histone H1 by cisplatin was isolated from the gel, purified and hybridized with 17mer primer, the sequence of which is complementary to the 3’ termini of 44mer templates (Figure 2), in the following way. The templates (5 \(\times\) 10\(^{-8}\) M) were annealed with 5’-end-radiolabeled 17mer primer at a molar ratio of 3:1 and incubated at 37°C in a volume of 50 μl in a buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM MgCl\(_2\),
Nucleotide excision repair (NER) assay

The 20mer oligonucleotides (the top and bottom strands of the duplex TGGT(NER), for its sequence, see Figure 1B) were used for preparation of linear 148-bp duplexes with centrally located DNA–protein CLs as described previously (18). Uniquely modified 20mers were annealed with a set of five complementary and partially overlapping oligonucleotides, and ligated with T4 DNA ligase. Full-length substrates were separated from unligated products in a 6% denaturing PAA gel, purified by electroelution, reannealed, and stored in annealing buffer [50 mM Tris–HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl2, and 1 mM DTT] at −20°C.

Duplexes containing single, site-specific adducts of cisplatin were then incubated with KF overnight and products were then resolved by denaturing 12% PAA/8 M urea gel and visualized by using a phosphor imager. Other details were published previously (15,17).

RESULTS

DNA–protein cross-linking by cisplatin or transplatin

Cisplatin and transplatin were investigated for their ability to form ternary DNA–protein complexes covalently linked to the platinum moiety. The proteins were chosen for these studies that bind to DNA with a relatively high affinity. KF− and the linker histone H1 were chosen as the representatives of non-sequence specific DNA-binding proteins with enzymatic or structural function, respectively, whereas transcription factor NF-κB (p50 dimer) was chosen as the representative of a sequence-specific DNA-binding protein with a regulation function. Also interestingly, histone H1 has been shown to bind more strongly to DNA modified by cisplatin than to non-modified DNA (21). The 40-bp duplex 5’-end-labeled at its top strand was globally modified by cisplatin or transplatin for 24 h so that 1, 2 and 4 platinum atoms were bound per duplex on average (r = 0.0125, 0.025 and 0.05, respectively). The duplex modified by cisplatin or transplatin (10 nM) was mixed with KF− or histone H1 (the molar ratio protein/duplex was 10). For the studies of the formation of ternary DNA–cisplatin–NF-κB complexes, the 20-bp duplex NF-κB (20) (its nucleotide sequence shown in Figure 1B corresponds to DNA consensus sequence of NF-κB) 5’-end-labeled at its top strand was used.

Ternary DNA–Pt–protein cross-linking efficiency was assessed by SDS/PAGE shift assay. Fractions were detected by SDS/PAGE with significantly retarded mobility (shown for r = 0.025 in Figure 2A–C, lanes 1–6) compared with that of the free probe (Figure 2A–C, lanes 7–10). The intensity of the bands with the retarded mobility increased with the incubation time and at a given time of this incubation (in the range of 1–24 h) it also increased with growing r value (not shown). These more slowly migrating fractions were eliminated after treatment with NaCN or protease K converting them to those of the unmodified probes (not shown). These results suggest that the species is a protein–DNA CL tethered by platinum–DNA and platinum–protein coordination bonds. While the protease K and NaCN experiments clearly indicate that protein is the species cross-linked to DNA, the amino acids participating in the cross-linking reaction have not been determined. Importantly, the amount of radioactivity associated with the bands corresponding to DPCLs formed by cisplatin was markedly higher than that by transplatin (cf. in Figure 2A–C, lanes 1–3 and 4–6) demonstrating that cisplatin exhibits a considerably higher efficiency to form ternary DNA–Pt–protein CLs than transplatin.
The yields of DNA–protein cross-linking by cisplatin using short 20- or 40-bp fragments were relatively low (Figure 2, Table 1). Therefore, we also used for similar studies DNA fragments 213-bp long. This fragment had a random nucleotide sequence so that we examined its cross-linking only to KF and histone H1 (since we had available no plasmid from which a similar DNA fragment containing the consensus nucleotide sequence for NF-κB protein could be obtained). The 213-bp duplex 3'-end-labeled at one strand was globally modified by cisplatin or transplatin for 24 h to the \( r_b \) value of 0.025. The fragment modified by cisplatin or transplatin (10 nM) was mixed with KF+ or histone H1 (the molar ratio protein/duplex was 10) and incubated overnight. Ternary DNA–Pt–protein cross-linking efficiency was assessed by 1% agarose (agarose and Metaphor agarose 1:1) gel shift assay. Similarly, as in the similar experiments using short duplexes, fractions were detected with significantly retarded mobility (Figure 3, lanes 1, 2, 5, 6) compared with that of the free probe (Figure 3, lanes 3, 4, 7–10). These more slowly migrating fractions were also eliminated after treatment with NaCN or proteinase K converting them to those of the unmodified probes (not shown). Importantly, the amount of radioactivity associated with the bands corresponding to DPCLs formed by cisplatin was markedly higher (~30% found for cross-linking of both histone H1 and KF+) than that obtained under similar conditions with short duplexes (~8–12%, Table 1). Moreover, the experiments with the 213-bp fragment also confirmed that cisplatin exhibits a considerably higher efficiency to form ternary DNA–Pt–protein CLs than transplatin (3 and 6% for cross-linking KF+ and histone H1, respectively).

Cisplatin and transplatin form various types of CLs and monofunctional adducts on DNA. Obvious candidates of DNA adducts of bifunctional platinum complexes that could preferentially bind proteins and form DPCLs are monofunctional adducts. Therefore, we examined the efficiency of monofunctional adducts of cisplatin to form DPCLs. We prepared 40-bp duplex globally modified by cisplatin. One sample was incubated with cisplatin at \( r_i = 0.025 \) (\( r_i \) is defined as the molar ratio of free platinum complex to nucleotide–phosphates at the onset of incubation with DNA) for 24 h; it implies that the resulting \( r_b \) was identical (0.025) since after this period all cisplatin molecules are bound to DNA quantitatively.

![Figure 3](image3.png)

**Figure 3.** Formation of DPCLs of unmodified and platinated 213-bp DNA fragment globally modified by cisplatin or transplatin \( (r_b = 0.025) \) with KF+ and histone H1 assessed by agarose gel electrophoresis; the fragment was incubated with the protein for 24 h. Lanes: 1, 2, the fragment modified by cisplatin incubated with KF+, histone H1, respectively; 3, 4, the fragment modified by cisplatin incubated in the buffer used for reaction with KF+, histone H1, respectively, no protein added; 5, 6, the fragment modified by transplatin incubated with KF+, histone H1, respectively; 7, 8, the fragment modified by transplatin incubated in the buffer used for reaction with KF+ or histone H1, respectively, no protein added; 9, 10, the unplatinated fragment incubated with KF+ or histone H1, respectively. See the text for other details.

![Figure 4](image4.png)

**Figure 4.** DPCL formation of unmodified and platinated oligodeoxyribonucleotide duplexes containing single, site-specific platinum adduct with KF+ assessed by SDS/PAA gel electrophoresis. Lanes: 1, 4, 7, 10, 13, the duplexes incubated with the protein for 4 h; 2, 5, 8, 11, 14 for 8 h; 3, 6, 9, 12, 15 for 24 h. Lanes: 1–3, the duplex TGT (20) containing monofunctional adduct of cisplatin; 4–6, the duplex TGT (20) containing monofunctional adduct of transplatin; 7–9, the duplex TGCT (20) containing 1,3-GTG intrastrand CL of cisplatin; 10–12, the duplex TGTT (20) containing 1,2-GG intrastrand CL of cisplatin; 13–15, the duplex TGCT (20) containing interstrand CL of cisplatin. See the text for other details.

| Time of incubation with the protein | DNA cross-linked to the protein (%) |
|-----------------------------------|-----------------------------------|
|                                  | KF+     | Histone H1 | NF-κB   |
|                                  | Cisplatin | Transplatin | Cisplatin | Transplatin | Cisplatin | Transplatin |
| 1                                 | 6.5 ± 0.1 | <0.1        | 3.5 ± 0.1 | 1.2 ± 0.1 | 0.5 ± 0.1 | 0          |
| 4                                 | 8.5 ± 0.2 | 0.5 ± 0.1   | 5.2 ± 0.1 | 1.6 ± 0.1 | 0.8 ± 0.1 | 0          |
| 24                                | 12.4 ± 0.2 | 0.8 ± 0.1   | 8.4 ± 0.2 | 1.9 ± 0.1 | 1.2 ± 0.1 | 0.3 ± 0.1   |

The duplexes were globally modified by cisplatin or transplatin \( (r_b = 0.025) \).

The duplex 40 bp was used in the experiments with KF+ and histone H1, whereas the duplex NF-κB (20) was used in the experiments with NF-κB (see Figure 1B for their nucleotide sequence).

Each value represents the average of four samples and standard errors are indicated.
The second sample of the same 40-bp duplex was modified at much higher \( r_i \) (0.038), but only for 1 h so that the resulting \( r_b \) value was also 0.025 [after this short period of reaction of cisplatin with DNA, only a fraction of the molecules of cisplatin coordinated to DNA bases (65%—this was verified by FAAS)]. Thus, we had two samples of 40-bp duplex, both contained the same amount of platinum adducts [two per duplex on average, this was verified using the procedures published previously (22,23)], but spectrum of the adducts in these two samples was different. Cisplatin and transplatin react with DNA in a two-step process (24). In the first step, cisplatin or transplatin forms monofunctional adducts which subsequently close to bifunctional CLs. Thus, the 40-bp duplex modified by cisplatin for only 1 h contained markedly more monofunctional adducts (55%) than this duplex platinated to the same level, but for a substantially longer time (24 h) (1–2%) (23).

These platinated 40-bp duplexes (5'-end-labeled at its top strand) were mixed with KF\(^-\) or histone H1 (the molar ratio protein/duplex was 10). Ternary DNA–Pt–protein cross-linking efficiency was assessed by SDS/PAGE shift assay (not shown). Quite surprisingly, the amount of radioactivity associated with the bands corresponding to DPCLs formed by cisplatin adducts in the duplex treated with cisplatin for only 1 h was even slightly smaller (1.2 \( \times \)) than in the duplex treated for 24 h.

As the latter duplex contained considerably less monofunctional adducts, it is reasonable to conclude that not only monofunctional adducts, but also at least some CLs formed by cisplatin can be efficiently transformed to DPCLs.

Therefore, further experiments were performed to determine which DNA CLs formed by cisplatin are not stable enough so that they can be transformed in the presence of DNA-binding proteins to DPCLs and with what efficiency. Short 20-bp oligonucleotide duplexes were prepared (for their sequence, see Figure 1B) containing single and central, site-specific adduct of cisplatin, namely 1,2-GG or 1,3-GTG intrastrand CLs, interstrand CL between guanine residues in the 5'-GC/5'-GC sequence, monofunctional adduct at the G residue in the TGT sequence and for comparative purposes also monofunctional adduct of transplatin at the G residue in the TGT sequence [the monofunctional adducts are very frequent and persisting adducts formed by this clinically ineffective platinum complex (25,26)]. These duplexes 5'-end-labeled at the top strand were incubated with KF\(^-\) or histone H1 under conditions used in the experiments with the 40-bp duplex (vide supra) and ternary DNA–Pt–protein cross-linking efficiency was assessed by SDS/PAGE shift assay (shown for cross-linking of KF\(^-\) in Figure 4). Importantly, 10% SDS/PAA gel electrophoresis cannot distinguish between 20mer single strand containing single intrastrand adduct of cisplatin and the 20-bp duplex interstrand cross-linked by this drug in contrast to 24% PAA/8M urea denaturing gel (Figure S2 in the Supplementary Material). Strong bands corresponding to the fractions with significantly retarded mobility (Figure 4, lanes 3, 9, 12) were only detected when the duplexes containing 1,2-GG, 1,3-GTG intrastrand CL and monofunctional adduct of cisplatin were examined. In contrast, only faint bands corresponding to the fraction with significantly retarded mobility were noticed for the duplex containing monofunctional adduct of transplatin (Figure 4, lanes 4–6), whereas no more slowly migrating band was noticed if the duplex containing interstrand CL of cisplatin was analyzed (Figure 4, lanes 13–15). It was verified that the more slowly migrating fractions were eliminated after treatment with NaCN or proteinase K converting them to those of the unmodified probes (not shown). Thus, only 1,2-GG, 1,3-GTG intrastrand CLs of cisplatin and monofunctional adduct of this drug can be transformed to DPCLs, whereas interstrand CLs of cisplatin and monofunctional adducts of transplatin are relatively stable in the presence of DNA-binding proteins so that they can be transformed to DPCLs only with markedly lower efficiency. Figure 4 also demonstrates that the amount of DPCLs increases with the time of the incubation of the duplexes with the proteins and that after 24 h the amount of the adduct transformed into DPCLs reaches 3.4, 3.2, 2.6 and 0.9% for 1,2-GG, 1,3-GTG intrastrand CL, monofunctional adduct of cisplatin and transplatin, respectively. It implies that intrastrand adducts of cisplatin can be transformed into the DPCLs with approximately identical efficiency, whereas the frequent monofunctional adducts of clinically ineffective transplatin much less efficiently.
It has been suggested by one of the reviewers that the transformation into DPCLs observed upon addition of DNA-binding proteins to the samples of 20-bp duplexes containing single 1,2-GG or 1,3-GTG intrastrand CL of cisplatin might be affected by the presence of small amounts (~1%) of other cisplatinated duplexes, such as the duplexes containing the monofunctional adduct or intrastrand CL involving platinated pyrimidine base(s). As we discuss in the Supplementary Material, this eventuality is unlikely.

**Inhibition of DNA polymerization**

Damage to DNA has significant effects on processivity of a number of DNA polymerases depending on the character of the damage (27,28). With DNA templates containing site-specifically placed adducts of various platinum compounds, a number of prokaryotic and eukaryotic DNA polymerases were blocked but could also traverse through platinum adducts depending on their character. It is, therefore, interesting to examine whether DPCLs will be processed by DNA polymerases in a different way than plain DNA intrastrand CLs of cisplatin (containing no proteins). We constructed the 17mer/44mer primer/template duplexes unplatinated or containing the 1,2-GG intrastrand CL of cisplatin in the central TGTT sequence (for their sequences, see Figure 5). The first 17 nt on the 3' terminus of the 44mer template strand were complementary to the nucleotides of the 17mer primer and the guanine involved in the 3' residue of the 1,2-GG intrastrand CL of cisplatin on the template strand was located at the 30th position from the 3' terminus (Figure 5). After annealing the 17mer primer to the 3' terminus of the unplatinated or platinated template strand positioning the 3'-end of the primer 15 bases before the adduct in the template strand, we examined DNA polymerization through the single, 1,2-GG intrastrand CL of cisplatin by RT HIV-1 in the presence of all four deoxyribonucleoside 5'-triphosphates. The reaction was stopped at various time intervals, and the products were analyzed using a sequencing 12% PAA/8 M urea gel (Figure 5).

Polymerization using the 44mer template containing the CL of cisplatin proceeded rapidly up to the nucleotide preceding and at the sites opposite the CL, such that mainly the 32, 33 and 34mer products accumulated to a significant extent (shown in Figure 5, lanes 6–9). The larger or shorter DNA intermediates were not observed in a considerable extent, whereas no intermediate products were seen with the 44mer control template as the full-length products were formed (shown in Figure 5, lanes 2–5). The full-length products were also noticed with the 44mer template containing the CL of cisplatin, although in a significantly smaller amount (Figure 5, lanes 6–9). This result is in agreement with a previously published work (15,17,29) and confirms that 1,2-GG intrastrand CL of cisplatin inhibits DNA synthesis, but translesion DNA synthesis may occur.

The 44mer template cross-linked to histone H1 by cisplatin was prepared from the template containing the 1,2-GG intrastrand CL of cisplatin to which histone H1 was added (vide supra). This template was isolated from the gel, purified, hybridized with 17mer primer and used as a substrate to investigate the translesion synthesis across the adducted nucleotide residues. As shown in the lanes 10–13 of Figure 5, polymerization by RT HIV-1 using the template cross-linked to histone H1 proceeded up to the nucleotide preceding the CL, such that mainly the 32nt products accumulated. Interestingly, DNA intermediates shorter than 32nt were also formed in a considerable extent, but no intermediates longer than 32nt or full-length products were observed. Hence, polymerization by RT HIV-1 using the template cross-linked to histone H1 was completely inhibited so that no translesion DNA synthesis occurred.

The lack of accumulation of intermediates shorter than those formed due to the termination of the polymerization at the site of the plain 1,2-GG intrasand CL or one nucleotide before this CL implies that the extension of all species ahead of the CL is faster than their rate of formation. The observation that these shorter intermediates are formed after this CL is transformed into the DPCL is consistent with the view that the DPCL considerably slows down the extension of the species ahead of this lesion. This may be due to its markedly increased bulkiness so that the lesion may interfere with DNA polymerization not only at the nucleotide to which the protein is covalently bound, but also at the preceding nucleotides.

**NER**

NER is a pathway used by human cells for the removal of damaged nucleotides from DNA (30). In mammalian cells, this repair pathway is an important mechanism for the removal of bulky, helix-distorting DNA adducts, such as those generated by various chemotherapeutics including cisplatin (31). Efficient repair of 1,2-GG or 1,3-GTG intrastrand CLs of cisplatin has been reported by various NER systems including human and rodent excinucleases (17,20,32). The results presented in Figure 6, lanes 2 and 3 are consistent with these reports. The major excision fragment contains 28 nt and other primary excision fragments are 24–29 nt in length (17,20,32). The 148-bp substrate cross-linked to KF− or histone H1 by cisplatin was prepared from the templates containing the 1,2-GG or 1,3-GTG intrastrand CL of cisplatin to which KF− or histone H1 was added (vide supra). These templates were isolated from the gel, purified and used as a substrate to investigate the removal of the DPCL formed by cisplatin by NER system. No removal of the cisplatin adduct cross-linked to KF− or histone H1 from the 148 bp substrate by human or rodent excinuclease was observed under conditions when the 1,2-GG or 1,3 GTG intrastrand adducts (not cross-linked to a protein) were readily excised (shown in Figure 6, lanes 4 and 5 for the substrate cross-linked to KF−).

**DISCUSSION**

In spite of the fact that the role of DNA adducts in the mechanism of antitumor effects of cisplatin and its
analogue has been extensively examined, the significance of the DPCLs in which DNA and proteins are covalently linked by these platinum complexes has not been always fully appreciated. Hence, the mechanism of the origin of these DPCLs has not been so far investigated in such details as other (plain) DNA adducts of platinum compounds (containing no protein). We show in this work that in cell-free media cisplatin forms DPCLs considerably more effectively than clinically ineffective transplatin (Figures 2 and 3 and Table 1). It implies that there is a positive correlation between the efficiency of mononuclear bifunctional platinum complexes to form DPCLs and their antitumor effects. Thus, the results of the present work are consistent with previous findings demonstrating that DPCLs persist longer in cells exposed to cisplatin than in those exposed to the chemotherapeutically inactive trans analogue (9,33,34), which suggests relevance of such lesions to antitumor effects of cisplatin.

In the present work, we paid attention to some aspects of the mechanism of the origin of the DPCLs by cisplatin, in particular to the mechanism of transformation of individual types of plain DNA adducts of cisplatin (and transplatin) into the DPCLs. We assumed initially that the DPCLs may originate mostly from monofunctional adducts of cisplatin. However, quite strikingly, the DPCLs also arise with a roughly identical efficiency by the transformation of DNA intrastrand CLs of cisplatin (Figure 4). In contrast, DNA interstrand CLs of cisplatin are markedly more stable in the presence of DNA-binding proteins than DNA intrastrand CLs of this metallodrug so that their transformation into DPCLs is markedly more difficult. This observation may be associated with a rather severe conformational distortion induced in DNA by interstrand CLs of cisplatin (35,36). The interstrand CL is preferentially formed by cisplatin between opposite guanine residues in the 5'-GC/5'-GC sequence (37). The cross-linked guanine residues are not paired with hydrogen bonds to the complementary cytosines, which are located outside the duplex and not stacked with other aromatic rings. All other base residues are paired, but distortion extends over at least 4 bp at the site of the CL. In addition, the cis-diammineplatinum(II) bridge resides in the minor groove and the double helix is locally reversed to a left-handed, Z-DNA-like form. This adduct induces the helix unwinding by 76–80° relative to B-DNA and also the bending of 20–40° of the helix axis at the cross-linked site toward the minor groove. If the DPCL is formed from DNA adducts of cisplatin, it is necessary for DNA-binding protein to come into a very close contact with DNA at the site of the platinum adduct and to bind to DNA at this site relatively strongly. This may not be easily achieved in the case of DNA heavily distorted by the interstrand CL of cisplatin in contrast to other, less distorting, DNA adducts of this drug.

It is also demonstrated in the present work that frequent monofunctional adducts of clinically ineffective transplatin are also transformed into DPCLs, but much less efficiently than intrastrand CLs or monofunctional adducts of cisplatin (Figure 4). This observation is consistent with the fact that in general monofunctional adducts of bifunctional transplatinum complexes undergo chelation reactions less readily than those of cisplatinum complexes (38).

It has been also demonstrated by others that the frequency of DPCLs in tumor cells treated with cisplatin is comparable with that of plain DNA interstrand CLs of this drug (33) or is even markedly higher (10). Thus, considering the bulky nature of DPCLs it is likely that these lesions formed by cisplatin represent a more distinct and persisting structural motif recognized by the components of downstream cellular systems processing DNA damage considerably differently than the plain DNA adducts of this metallodrug (39). This conclusion is corroborated by the results of the present work (Figures 5 and 6) demonstrating that DPCLs linked by cisplatin inhibit DNA polymerization or removal of major cisplatin adducts from DNA by NER systems much more efficiently than the plain DNA adducts (not linked to proteins).

Our observation (Figure 6) that the mammalian NER system did not remove proteins cross-linked to DNA at a detectable level deserves further discussion.

Figure 6. Excision of the adducts of platinum complexes by rodent excinuclease. The 148 bp substrates were incubated with CHO AA8 CFE and subsequently treated overnight with NaCN prior to analysis in 10% PAA/8 M urea denaturing gel. Lanes: 1, control, unplatinated substrate; 2, the substrate containing 1,3-GTG intrastrand CL of cisplatin; 3, the substrate containing 1,2-GG intrastrand CL of cisplatin; 4, the substrate containing single DPCL formed by the transformation of the template containing site-specific 1,3-GTG intrastrand CL of cisplatin incubated with KF⁻; 5, the substrate containing single DPCL formed by the transformation of the template containing site-specific 1,2-GG intrastrand CL of cisplatin incubated with KF⁻. See the text for other details.
This phenomenon was first observed by us 3 years ago (15). We investigated in this recent work excision by mammalian repair systems of the DNA–protein CL formed by another antitumor platinum drug, trans-[PtCl2(E-iminoether)2], between 148 bp DNA fragment and 20 kDa histone H1 and found that such bulky DNA–protein CLs were not removed. Quite recently, our earlier results have been confirmed by others (40) demonstrating that the human NER system did not remove other, also bulky 16 kDa protein (T4 pyrimidine dimer glycosylase) cross-linked to DNA. It has been also suggested (40) that the failure to remove DNA–bulky protein CLs is due to the steric hindrance caused by the size of the cross-linked protein that may interfere sterically with the assembly of the mammalian excision system. Thus, our results demonstrating failure of the mammalian NER system to remove the DNA–Pt–protein CLs reinforce the view that DNA–protein CLs formed by platinum drugs may be among the critical lesions relevant to their antitumor effects. Other proteins known to specifically bind to cisplatin–DNA lesions and proteins involved in damage recognition and repair signaling will be used in our future research to obtain more details on processing DNA damage by cisplatin in vivo.

The results of the present work also demonstrate that in cell-free media transplatin forms less DPCls than cisplatin (Figures 2 and 3). This result may seem to contradict conclusions published earlier by others according to which transplatin forms considerably more DPCls than cisplatin (9,33). However, these earlier conclusions have been drawn on the basis of the results obtained with the cells treated with equitoxic doses of cisplatin and transplatin, demonstrating a much higher molar concentration for transplatin. Thus, if these results are normalized to the identical level of DNA platination, then in accordance with the results of the present work transplatin is much less effective agent capable of forming DPCls than cisplatin. It is generally assumed that cytotoxic DNA adduct of antitumor drug has to be fairly persistent (41). Among DNA adducts of cisplatin this requirement is very well accomplished by DPCls. Hence, a further systematic and detailed analysis of the efficiency of cisplatin and other antitumor platinum compounds to form DPCls in cells and the role of these lesions in the mechanism underlying antitumor effects of platinum drugs is urgently needed.

There are several reactive agents which produce CLs between DNA and proteins in van der Waals’ contact. Thus, the cross-linking procedures involving these agents provide a tool for identification of proteins or protein domains closely positioned to DNA including mapping of protein-binding sites on DNA in vitro (42). The results of the present work support the view that cross-linking DNA and proteins by platinum complexes can be also applied to studies of specific protein–DNA interactions both in vitro and in vivo (43).

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

ACKNOWLEDGEMENTS

This research was supported by the Grant Agency of the Czech Republic (Grants 305/05/2030 and 204/03/H016), the Grant Agency of the Ministry of Health of the Czech Republic (NR562-4/2005), Ministry of Education of the CR (MSMT LC06030) and the Academy of Sciences of the Czech Republic (Grants 1QS500040581 and KAN200200651). J.K. is the international research scholar of the Howard Hughes Medical Institute. K.C. was supported by a doctoral fellowship from the Faculty of Sciences, Masaryk University, Brno. The authors also acknowledge that their participation in the EU COST Actions D20 (Grants MSMT CR 1P05OC070, 1P05OC072) enabled them to exchange regularly the most recent ideas in the field of platinum anticancer drugs with several European colleagues. Funding to pay the Open Access publication charge was provided by the Academy of Sciences of the Czech Republic.

Conflict of interest statement. None declared.

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