

# Mechanistic studies of the modulation of cleavage activity of topoisomerase I by DNA adducts of mono- and bi-functional Pt<sup>II</sup> complexes

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## ABSTRACT

**Using electrophoresis and replication mapping, we show that the presence of DNA adducts of bifunctional antitumor cisplatin or monodentate [PtCl(dien)]Cl (dien = diethylenetriamine) in the substrate DNA inhibits eukaryotic topoisomerase 1 (top1) action, the adducts of cisplatin being more effective. The presence of camptothecin in the samples of platinated DNA markedly enhances effects of Pt–DNA adducts on top1 activity. Interestingly, the effects of Pt–DNA adducts on the catalytic activity of top1 in the presence of camptothecin differ depending on the sequence context. A multiple metallation of the short nucleotide sequences on the scissile strand, immediately downstream of the cleavage site impedes the cleavage by top1. On the other hand, DNA cleavage by top1 at some cleavage sites which were not platinated in their close proximity is notably enhanced as a consequence of global platination of DNA. We suggest that this enhancement of DNA cleavage by top1 may consist in its inability to bind to other cleavage sites platinated in their close neighborhood; thus, more molecules of top1 may become available for cleavage at the sites where top1 normally cleaves and where platination does not interfere.**

## INTRODUCTION

Platinum-based drugs such as cisplatin, carboplatin, oxaliplatin and nedaplatin are clinically used against various solid tumors including genitourinary, colorectal and non-small cell lung cancers (1). The clinical use of antitumor platinum drugs, however, is restricted since many tumor cells display inherent or acquired resistance to platinum-based drugs, other limitations are due to dose-limiting side effects (2). Continuous efforts have been made to

overcome these limitations with a primary focus on the development of new platinum drugs. Designing new antitumor platinum drugs depends on understanding details of molecular and biochemical mechanisms associated with the biological effects of the existing platinum agents. Therefore, studies focused on understanding complex mechanisms underlying antitumor activity of platinum drugs represent now very active research area. It is generally accepted that the main biological event that triggers the anticancer properties of the platinum drugs is formation of platinum–DNA adducts (2–4).

Studies focused on mechanisms of antitumor activity of cisplatin have revealed an interesting phenomenon demonstrating that the frequency of the individual adducts formed on DNA by antitumor platinum compounds (which may differ in their biological effects) is dependent on DNA topology (5,6). These observations insinuate hypothesis that the mechanism of biological activity of platinum drugs could be also affected by catalytic efficiency of topoisomerases.

Topoisomerases are ubiquitous and vital enzymes because of their role in the control of the topological state of DNA. As they alter the topology of DNA, topoisomerases participate in nearly all events related to DNA metabolism including replication, transcription and recombination. The topoisomerases are now viewed as important therapeutic targets for cancer therapy; in particular, topoisomerase inhibitors are considered promising anticancer agents (7–9). Hence, it is somewhat surprising that the information on the nature of direct effects of cisplatin on topoisomerase catalytic activity is relatively rare.

Cisplatin inhibits topoisomerase II leading to a transient alteration of DNA supercoiling (10). *In vitro* studies of the effect of cisplatin on the supercoiling activity of purified topoisomerase II have revealed that cisplatin is an efficient inhibitor of this enzyme as a consequence of a direct interaction of cisplatin with the enzyme. On the other hand, the results of several reports (11,12) are consistent with the hypothesis that topoisomerase

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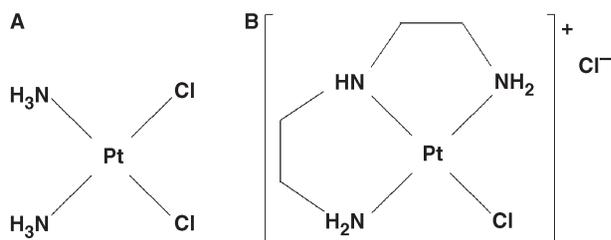


Figure 1. Chemical structures of cisplatin (A) and [PtCl(dien)]Cl (B).

catalytic activity is also affected by DNA adducts of cisplatin. For instance, a synergism between cisplatin, oxaliplatin or nedaplatin and several topoisomerase I (top1) inhibitors in inhibiting its catalytic activity has been demonstrated *in vitro* (11,13) and in human small cell lung cancer cells (13–16). It is reasonable to assume that severe conformational alterations induced in DNA by the crosslinks of cisplatin and its analogs modulate the stabilization of top1-drug-DNA cleavable complexes and/or as topoisomerase inhibitors may inhibit repair of cisplatin adducts (12,14) so that the antitumor activity of the platinum drug is enhanced. Also interestingly, the reaction of closed circular DNA containing cisplatin adducts with top1 yields unique topoisomers (17). In addition, it has been shown (18) that 1,3-GTG intrastrand crosslinks [which represent only very minor DNA adducts of cisplatin (19)] formed by this metallodrug in 24-bp oligonucleotide containing no preferential recognition site of top1, enhance DNA cleavage by top1 in the presence of top1 poison such as camptothecin (CPT). Hence, further investigations of the effect of DNA adducts of platinum compounds on the catalytic activity of topoisomerases are warranted to define their role in the mechanism of the cytotoxicity of these metallodrugs.

The purpose of this work is to determine whether activity of eukaryotic top1 is affected by the modification of DNA by antitumor cisplatin. Positive results may represent a new alternative concept for the mechanism, effective in some types of tumors, underlying antitumor activity of transition metal-based drugs. We selected two complexes to investigate the effects of platinum adducts formed in DNA on the activity of top1; bifunctional cisplatin (Figure 1A) and ineffective monodentate [PtCl(dien)]Cl (dien = diethylenetriamine) (Figure 1B) frequently used in the studies of structure–pharmacological activity relationship of platinum compounds (20). Bidentate cisplatin forms on the DNA major intrastrand adducts between two adjacent purine residues (~90%), mainly such as 1,2-GG intrastrand crosslinks of adjacent deoxyriboguanosines with platinum binding the N7 positions of bases. These crosslinks are believed to be at the origin of antitumor activity of cisplatin and its analogs (1–4). It has been demonstrated that formation of 1,2-GG intrastrand crosslink by cisplatin leads to marked conformational alterations in DNA structure including unwinding the DNA duplex in the vicinity of the site of platination, bending it toward the major groove and generating a widened and shallow minor groove (2). Owing to the fact that top1 binds specifically to the sites in DNA

that contain a number of preferential binding sites of cisplatin and its analogs (21–23) it seems reasonable to expect that such a severe distortion of DNA or just metalation *per se* within top1 DNA binding site affects activity of this enzyme. On the other hand, [PtCl(dien)]Cl forms in DNA monofunctional adducts which induce only minor changes in DNA structure so that it is of interest to compare the effects of cisplatin adducts on activity of top1 with those of less distorting adducts of [PtCl(dien)]Cl.

## MATERIALS AND METHODS

### Starting materials

Plasmid pSP73KB [2455 bp, supercoiled density  $\sigma = -0.063$ ] was isolated according to standard procedures. Phagemid pBluescript SK(–) (2958 bp,  $\sigma = -0.058$ ) was purchased from Stratagene (La Jolla, CA, USA). PvuII and HindIII restriction endonucleases, T4 polynucleotide kinase and Circum Vent™ Thermal Cycle Sequencing Kit with Vent(exo<sup>-</sup>) DNA polymerase were purchased from New England Biolabs (Beverly, MA, USA). The 161-bp DNA fragment was prepared by digesting supercoiled pBluescript SK(–) phagemid with PvuII and HindIII restriction endonucleases in the NEB buffer 2 supplied by the manufacturer and separated by electrophoresis in a 1% agarose gel made in 1× TBE buffer. The 161-bp fragment was isolated from the gel by Promega Wizard SV Gel clean-up system. Approximately 100 ng of the fragment was 3′-end-labeled at the HindIII site by fill-in reaction with [ $\alpha$ -<sup>32</sup>P]-dATP and 0.5 mM dGTP, dCTP and dTTP, in the EcoPol buffer supplied by the manufacturer with 1 unit of the Klenow fragment from DNA polymerase I (exonuclease minus, mutated to remove the 3′–5′ proofreading domain) (KF<sup>-</sup>). Unincorporated [ $\alpha$ -<sup>32</sup>P]-dATP was removed by Promega Wizard SV Gel clean-up system, and the eluate containing the 3′-end-labeled 161-bp fragment was collected, freeze dried and redissolved in 40  $\mu$ l of 10 mM Tris–HCl, pH 7.4. The synthetic oligodeoxyribonucleotides were purchased from DNA Technology A/S (Arhus, Denmark). The DNA top1 from calf thymus, KF<sup>-</sup> and bovine serum albumin (BSA) were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). [ $\alpha$ -<sup>32</sup>P]-dATP and [ $\gamma$ -<sup>32</sup>P]-ATP were from MP Biomedicals, LLC (Irvine, CA, USA). Acrylamide, bis(acrylamide) and ethidium bromide (EtBr) were from Merck KgaA (Darmstadt, Germany). Agarose was from FMC BioProducts (Rockland, ME, USA). Wizard SV Gel and PCR Clean-Up System used to extract and purify 161-bp DNA fragment (*vide infra*) were purchased from Promega (Madison, WI, USA). CPT, NaCN, dimethyl sulfoxide (DMSO), dimethyl sulfate (DMS) and cisplatin were from Sigma-Aldrich (Prague, Czech Republic). SDS was from Serva (Heidelberg, Germany). CPT was dissolved in DMSO at 10 mM concentration and stored at –20°C. [PtCl(dien)]Cl was a kind gift of Prof. G. Natile from University of Bari.

### Platination reactions

Plasmid DNA or the 161-bp fragment were incubated with the platinum complex in 10 mM NaClO<sub>4</sub> at 37°C for 24 h

in the dark if not stated otherwise. The number of molecules of the platinum compound bound (coordinated) per nucleotide residue ( $r_b$  value) was determined by flameless atomic absorption spectrophotometry (FAAS). It was verified that under these conditions of DNA platination, no free molecules of the platinum complex remained in the samples further used in the experiments described in this work.

The oligonucleotides were allowed to react with the platinum compounds, and repurified as described previously (24,25). The single-stranded oligonucleotide (the top strand in Figure 6A) was reacted in stoichiometric amounts with cisplatin or [PtCl(dien)]Cl. The platinated oligonucleotides were purified by FPLC. It was verified by platinum FAAS and by the measurements of the optical density that the modified oligonucleotides contained one platinum atom. It was also verified using DMS footprinting of platinum on DNA (25,26) that in the platinated top strands the N7 position of both neighboring guanines for cisplatin or one of the guanine residues at +1 or +2 position for [PtCl(dien)]Cl was not accessible for reaction with DMS. Briefly, platinated and nonmodified top strands (5'-end-labeled with  $^{32}\text{P}$ ) were reacted with DMS. DMS methylates the N7 position of guanine residues in DNA producing alkali labile sites (27). However, if N7 is covalently bound to platinum, it cannot be methylated. The oligonucleotides were then treated with hot piperidine and analyzed by denaturing polyacrylamide (PAA) gel electrophoresis. For the nonmodified oligonucleotides, shortened fragments due to the cleavage of the strand at the two methylated guanine residues were observed in the gel. However, no such bands were detected for the platinated oligonucleotides. These results indicate that one cisplatin or [PtCl(dien)]Cl molecule was coordinated to both or one of the neighboring guanine residues, forming the 1,2-GG intrastrand crosslink or monofunctional adduct. FPLC purification was carried out on a Pharmacia Biotech FPLC System with MonoQ 5/50 GL.

Thus, all experiments described in the present work were performed in the absence of free (unbound) platinum complex present in the reaction mixtures containing top1. In this way, any inhibition of top1 catalytic activity due to a direct modification of top1 by the platinum complexes was excluded.

#### Top1-catalyzed relaxation of negatively supercoiled DNA

Each reaction mixture (40  $\mu\text{l}$ , total volume) contained 2  $\mu\text{g}$  of supercoiled pSP73(KB) plasmid DNA nonmodified or globally modified by cisplatin or [PtCl(dien)]Cl in 35 mM Tris-HCl, pH 8.0, 72 mM KCl, 5 mM  $\text{MgCl}_2$ , 5 mM DTT, 5 mM spermidine, 0.01% BSA and  $\sim 4$  pM top 1. Some reaction mixtures contained 100  $\mu\text{M}$  CPT. The order of addition was DNA, buffer, CPT and top 1. The reaction mixtures were incubated at 37°C, and at indicated times 9  $\mu\text{l}$  aliquots were withdrawn and the reaction terminated by the addition of 0.5  $\mu\text{l}$  of 10% SDS. All samples were then mixed with the loading buffer and loaded onto a 1% agarose gel running at 25°C in the dark with TAE (Tris-acetate/EDTA) buffer and the voltage set at 25 V. The gels were then stained with EtBr, followed by

photography with transilluminator. The bands were quantitated by integration with the AIDA image analyzer software (Raytest, Germany). Plasmid DNA relaxation mediated by top1 was determined by quantitating the loss of supercoiled plasmid DNA having original supercoiled density  $\sigma = -0.063$  as a function of time. It was verified that under the conditions employed, the band intensity in the negative of the gel photograph was directly proportional to the amount of DNA present.

#### Top1-mediated cleavage of the 161-bp DNA fragment

Aliquots of the 161-bp fragment [PvuII/HindIII fragment of pBluescript SK(-) phagemid] were modified by cisplatin or [PtCl(dien)]Cl to various values of  $r_b$  and then allowed to react with top1 at 20°C for 30 min in the absence or presence of surplus of CPT. Each reaction (5  $\mu\text{l}$ ) contained top1 at the concentration of  $\sim 50$  pM, the 161-bp fragment at the concentration of  $\sim 1.5$  nM and CPT at the concentration of 5  $\mu\text{M}$ . Reactions were terminated by adding SDS (0.5% final concentration) and each sample was divided into two halves. One half was mixed with formamide loading buffer and analyzed on 13% PAA/7 M urea denaturing gel. The other half was incubated with 0.3 M NaCN (pH 11) at 45°C for 10 h in the dark to remove platinum complexes from DNA. Samples were then precipitated by ethanol, redissolved in formamide loading buffer and analyzed on a denaturing PAA gel. The autoradiograms were visualized and quantified by using the bio-imaging analyzer.

#### Sequence preference of Pt-DNA adducts in the 161-bp DNA fragment

The primer extension assay was used to evaluate the sequence selectivity of DNA modification by cisplatin and [PtCl(dien)]Cl. The 161-bp fragment [from pBluescript SK(-) phagemid, *vide supra*] was incubated with platinum complexes in 10 mM  $\text{NaClO}_4$  for 24 h at 37°C to obtain  $r_b = 0.005$  or 0.008. The excess of drug was removed by ethanol precipitation. Circum Vent™ Thermal Cycle Sequencing Kit with Vent(exo<sup>-</sup>) DNA polymerase was used along with the protocol for thermal cycle DNA sequencing recommended by the manufacturer with small modifications (28) with 5'-end-labeled 18-mer primers (5'-CTGGCGAAAGGGGGATGT complementary to the sites 1–18 in the bottom strand and 5'-TATC GATACCGTCGACCT complementary to the sites 140–157 in the top strand of the 161-bp fragment). The synthesis products were separated by electrophoresis on a denaturing PAA gel (6% PAA/8 M urea); sequence ladders were obtained in parallel using untreated control DNA fragment.

#### Top1-mediated cleavage of the 30-bp oligonucleotide duplex

The upper strand of the duplex (its sequence is shown in Figure 6A) unplatinated or containing single, site-specific platinum adduct was annealed with unplatinated complementary strand (the bottom strand of the duplex shown in Figure 6A) in 50 mM NaCl, 10 mM Tris-HCl with 0.1 mM EDTA (pH 7.4) and 3'-end-labeled with

$[\alpha\text{-}^{32}\text{P}]\text{-dATP}$  and  $\text{KF}^-$ . Unincorporated  $[\alpha\text{-}^{32}\text{P}]\text{-dATP}$  was removed by G50 Sephadex column. Each reaction (5  $\mu\text{l}$ ) contained DNA substrates ( $\sim 5\text{ nM}$ ) which were incubated with  $\sim 60\text{ pM}$  top1 for 30 min at  $20^\circ\text{C}$  in the presence or absence of  $10\text{ }\mu\text{M}$  CPT in  $10\text{ mM}$  Tris-HCl, pH 7.5,  $50\text{ mM}$  KCl,  $5\text{ mM}$   $\text{MgCl}_2$ ,  $0.1\text{ mM}$  EDTA and  $15\text{ }\mu\text{g/ml}$  BSA. Reactions were stopped by addition of  $0.5\%$  SDS, mixed with formamide loading buffer and analyzed on a  $24\%$  PAA/7M urea denaturing gel.

### Other physical methods

Absorption spectra were measured with a Beckman DU 7400 spectrophotometer equipped with a thermoelectrically controlled cell holder and quartz cells with the path length of 1 cm. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. For FAAS analysis, DNA was precipitated with ethanol and dissolved in  $0.1\text{ M}$  HCl. The gels were visualized by using a BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivity associated with bands was quantitated with the AIDA image analyzer software (Raytest, Germany).

## RESULTS

### Inhibition of top1-mediated DNA relaxation

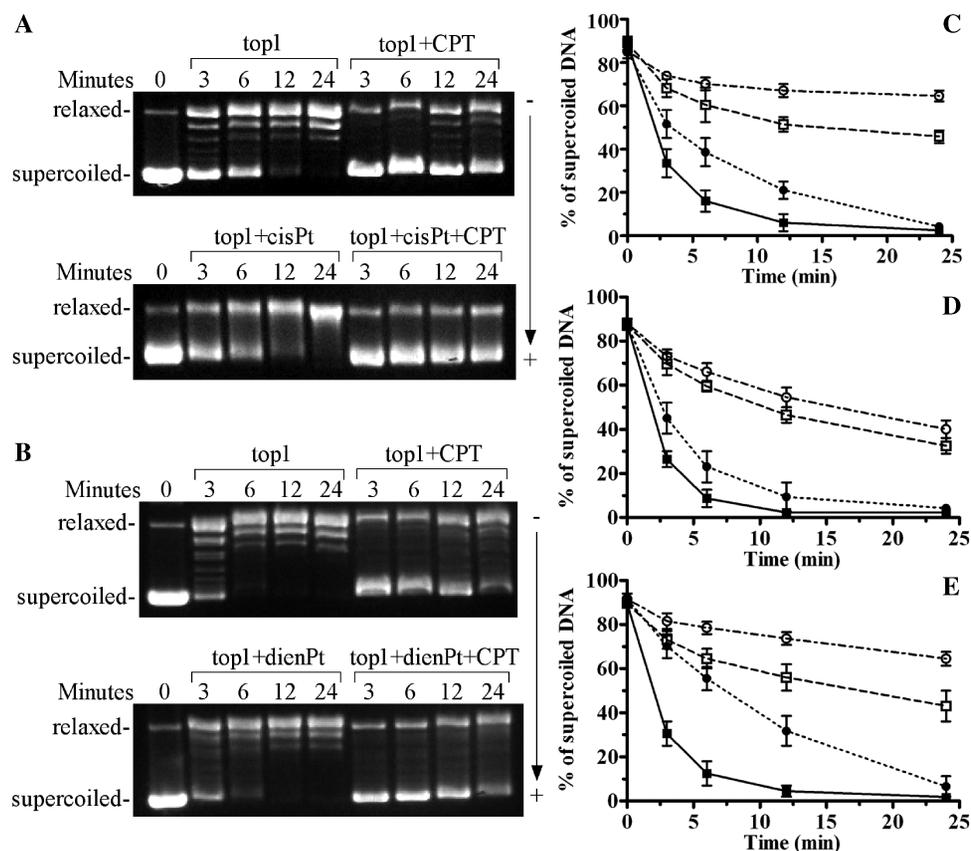
A DNA relaxation assay was utilized to examine the effects of damage induced in the substrate by adducts of platinum complexes on the catalytic activity of eukaryotic DNA top1 and how CPT affects top1-DNA interactions under such conditions. This assay measured the conversion of naturally negatively supercoiled pSP73KB plasmid into relaxed covalently closed circular DNA. The effect of DNA platination on top1 catalyzed DNA relaxation was assessed by comparing the rate of relaxation of unplatinated supercoiled plasmid with plasmid globally modified by bidentate cisplatin (Figure 2A) or monodentate  $[\text{PtCl}(\text{dien})]\text{Cl}$  (Figure 2B) in the absence or presence of CPT. The level of platination of DNA could not be too high because both platinum complexes, cisplatin in particular, unwind the DNA (29), which may complicate analysis of the bands in gels at higher levels of platination. The highest  $r_b$  value used was 0.02 or 0.04 in the case of cisplatin or  $[\text{PtCl}(\text{dien})]\text{Cl}$ , respectively. The time course of relaxation of plasmid DNA modified by cisplatin or  $[\text{PtCl}(\text{dien})]\text{Cl}$  by top1 in the presence and absence of CPT is shown in Figure 2C-E. Modification of DNA by either complex reduced the rate at which top1 relaxed supercoiled form of DNA. Cisplatin is more efficient than  $[\text{PtCl}(\text{dien})]\text{Cl}$  at  $r_b = 0.02$ , nevertheless after  $\sim 20$  min the intensities of the bands corresponding to supercoiled DNA were in both cases reduced to zero. Comparison of the plots shown in Figure 2D and E reveals that the rate of relaxation of DNA by top1 decreases with growing level of DNA platination.

CPT and its derivatives belong to efficient inhibitors of top1 catalytic activity which have recently been introduced in cancer treatment. CPT inhibits the religation step. When CPT was present in the samples containing

DNA modified by the platinum complexes, resulting inhibitory effects on top1 activity were rather additive than synergistic (Figure 2). Interestingly, some more than additive effects as regards the inhibition of top1 activity of CPT and platinum adducts are observed at the longest times of the reaction (24 min) when inhibition caused by platination in the absence of CPT is nearly zero.

### Top1-mediated cleavage of 161-bp DNA fragment

Top1 cleaves DNA at multiple sites, but selectively at certain nucleotide sequences (21–23,30). Thus, the other way to determine top1 catalytic activity involves an analysis of the DNA cleavage pattern. We examined influence of DNA adducts of cisplatin or  $[\text{PtCl}(\text{dien})]\text{Cl}$  on top1-mediated cleavage in the 161-bp 3'-end-labeled PvuII/HindIII fragment of pBluescript SK(-) phagemid DNA. This fragment contains several sequences corresponding to top1 specific and preferential recognition sites (31). First, we examined the cleavage in the absence of CPT, but top1 did not produce detectable cleavage although an amount of top1 considerably higher than in the DNA relaxation assay was used (data not shown). Thus, CPT was required for observing the expected 161-bp duplex cleavage products (Figure 3, lanes, control) so that we examined effect of DNA adducts of the platinum complexes on top1-mediated cleavage in the presence of CPT. CPT has been shown to stimulate DNA cleavage by top1 at its recognition sequences although it enhances cleavage at some sites much more than at others. Moreover, it does not alter significantly the sequence specificity of enzyme-mediated cleavage (30). The sites of top1-mediated single-strand cleavage produced by CPT on a 161-bp fragment are shown in Figure 3 (lanes control). Five major sites of cleavage can be clearly seen; the sequences at which the major cleavage occurs are displayed on the right side of the Figure 3B. The guanines as preferential binding sites of cisplatin and  $[\text{PtCl}(\text{dien})]\text{Cl}$  are highlighted as bold letters. An examination of the gels reveals that with growing level of the modification of the fragment by cisplatin (Figure 3A) or  $[\text{PtCl}(\text{dien})]\text{Cl}$  (Figure 3B), cleavage induced by CPT was affected at some cleavage sites as evident from changes of intensities of some bands corresponding to the cleavage fragments. Figure 4 demonstrates the dependence of the DNA cleavage mediated by top1 in the presence of CPT on the extent of modification of the DNA by cisplatin (Figure 3A) or  $[\text{PtCl}(\text{dien})]\text{Cl}$  (Figure 3B). DNA cleavage at the sites in the 37 and 119 positions was strongly suppressed with growing global modification of the fragment by both cisplatin and  $[\text{PtCl}(\text{dien})]\text{Cl}$ , cisplatin being more effective (Figure 4). On the other hand, DNA cleavage at the sites corresponding to the 92 and 97 positions was notably enhanced in particular at lower levels of modification by  $[\text{PtCl}(\text{dien})]\text{Cl}$  and cisplatin, respectively. The cleavage of the 161-bp fragment modified by cisplatin was not affected or was affected weakly at the sites corresponding to the 70 and 92 positions, respectively; when the fragment was modified by  $[\text{PtCl}(\text{dien})]\text{Cl}$  the cleavage was unaffected or affected weakly at the sites corresponding to 70 and 97 positions.



**Figure 2.** Inhibition of top1-mediated relaxation of negatively supercoiled pSP73KB plasmid by cisplatin or [PtCl(dien)]Cl adducts in the absence or presence of CPT analyzed by agarose gel electrophoresis. (A and B) Electrophoretograms of nonmodified plasmid (top gels in panels A and B) and plasmid modified by cisplatin (A) or [PtCl(dien)]Cl (B) to  $r_b = 0.02$  (bottom gels in panels A and B) were treated with top1 in the absence or presence of 100  $\mu$ M CPT. Sets of lanes in panels A and B represent increasing reaction times in minutes before termination of the reaction by 0.5% SDS; samples were then mixed with loading buffer and electrophoresed. (C–E) Plots of plasmid DNA relaxation mediated by top1 (see panels A and B). At the indicated times, aliquots were removed, the reaction was terminated with 0.5% SDS, electrophoresed and the percentage of supercoiled form of DNA was determined by densitometry. Filled square and open square correspond to unplatinated supercoiled plasmid treated with top1 in the absence and presence of 100  $\mu$ M CPT, respectively. Filled circle and open circle correspond to supercoiled plasmid modified by cisplatin to  $r_b = 0.02$  (C) or [PtCl(dien)]Cl to  $r_b = 0.02$  (D) and 0.04 (E) treated with top1 in the absence and presence of CPT, respectively.

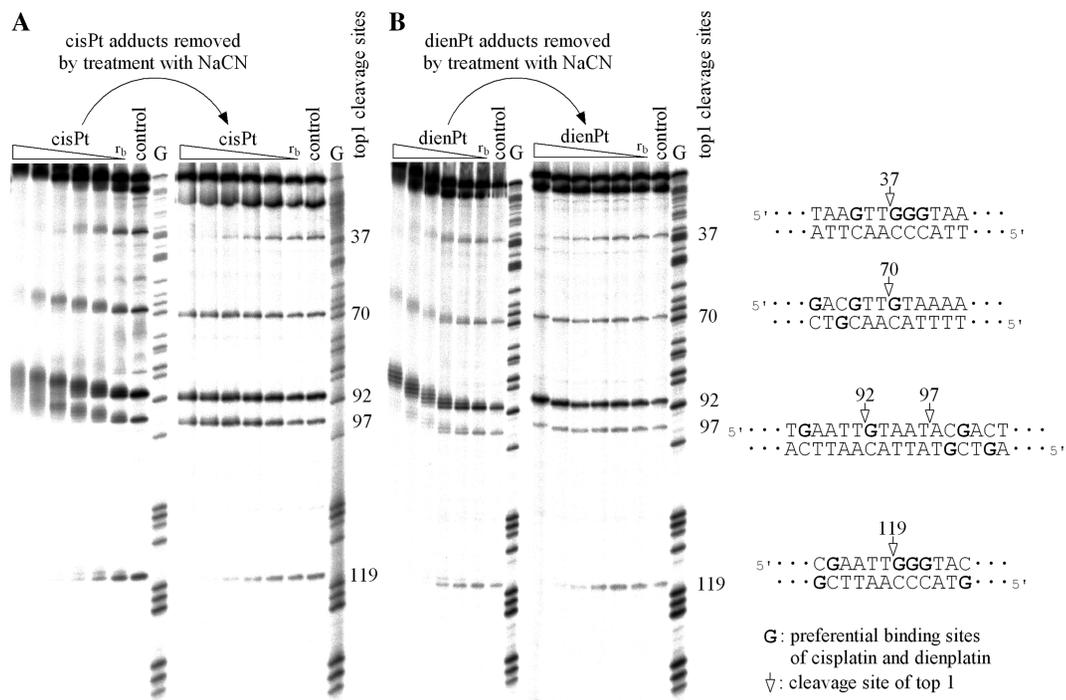
### Replication mapping of platinum adducts in 161-bp DNA fragment

This procedure involved the extension by VentR(exo–) DNA polymerase at the 3'-end of the primer up to the metal adduct on the template strand of PvuII/HindIII fragment of pBluescript SK(–) phagemid DNA. The products of the synthesis were then examined on DNA sequencing gels, and the sequence specificity of the platinum adduct formation was determined to the exact nucleotide. *In vitro* DNA synthesis on DNA templates containing the adducts of cisplatin or [PtCl(dien)]Cl generated a population of DNA fragments, indicating that the adducts of these complexes terminate DNA synthesis (Figure 5A, lanes cisPt1,2 and dienPt1,2). In general, intensity of the bands corresponding to the termination of DNA synthesis due to the adducts of [PtCl(dien)]Cl was weaker than that due to the adducts of cisplatin. Sequence analysis of the termination sites produced by cisplatin and [PtCl(dien)]Cl confirmed a strong preference of their DNA binding to guanine residues (Figure 5B). Interestingly, intense platination by both cisplatin and

[PtCl(dien)]Cl occurred at three consecutive G residues (sites 38–40 and 120–122) on the scissile strand of the 161-bp fragment. These sites are immediately downstream of the cleavage sites 37 and 119, respectively at which top1 normally cleaves DNA both in the presence and absence of CPT. Other residues in the close proximity of the cleavage site of top1 on the scissile strand was that at the guanine residue 93, i.e. immediately downstream of the cleavage site 92. This G residue (93) was, however, metallated only by cisplatin (Figure 5). Also interestingly, the single A residue 98 immediately downstream of the cleavage site 97 was also metallated, but mainly by [PtCl(dien)]Cl (Figure 5). Other platinated sites in the 161-bp fragment modified by cisplatin or [PtCl(dien)]Cl in the immediate neighborhood of the cleavage sites of top1 on the scissile strand were not observed (Figure 5).

### Top1-mediated cleavage of the 30-bp oligonucleotide duplex

The influence of major adducts of cisplatin and [PtCl(dien)]Cl on the activity of eukaryotic top1 was

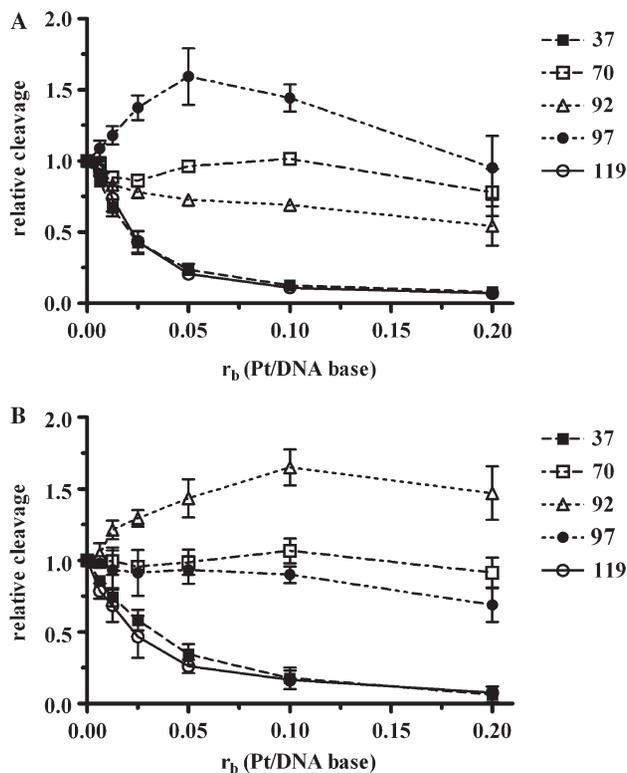


**Figure 3.** Effect of DNA modification by cisplatin (A, lanes cisPt) or [PtCl(dien)]Cl (B, lanes dienPt) on top1-mediated cleavage in the presence of CPT. A 161 bp 3'-end labeled DNA fragment modified by cisplatin or [PtCl(dien)]Cl at  $r_b = 0.00625, 0.0125, 0.025, 0.05, 0.1$  and  $0.2$  was treated with top1 in the presence of  $5 \mu\text{M}$  CPT. After 30 min, reactions were terminated by addition of SDS and the sample was split into two parts. One half of the sample was analyzed immediately (gels on the left of each panel) by PAA electrophoresis under denaturing conditions and the other half (gels on the right of each panel) was first incubated with NaCN to remove platinum adducts and only then electrophoresed. Lanes control, nonmodified DNA treated with top1 in the presence of  $5 \mu\text{M}$  CPT; lanes G correspond to Maxam–Gilbert G ladder. DNA sequences of the five major cleavage sites mediated by top1 in the presence of CPT are shown on the right of (B). The arrow indicates the position of the DNA single-strand breaks and guanines as potential preferential binding sites of cisplatin and [PtCl(dien)]Cl are highlighted as bold letters.

also examined using short DNA oligonucleotide (30 bp) containing a unique top1 cleavage site (22,32,33) (Figure 6A). Top1 cleaves one strand of double-helical DNA between T and G(+1)G(+2) bases in the 5'-CTTG(+1)G(+2)A- sequence derived from the oligonucleotide used for determination of the crystal structure of human top1 bound to this DNA substrate (34,35) (Figure 6B and C). The presence of two adjacent guanine bases made it possible to prepare on the scissile strand at the +1 and +2 positions downstream from the top1 scissile bond the most frequent adduct of cisplatin, 1,2-GG intrastrand crosslink, which is believed to be responsible for its antitumor activity. It seems interesting to test whether extensive distortions induced in DNA by formation of the 1,2-GG intrastrand crosslink within top1 binding site affect activity of top1. Indeed, cleavage of the duplex by top1 is completely inhibited by formation of cisplatin adduct (Figure 6B, lane 4) and no cleavage products are observed even in the presence of CPT (Figure 6B, lane 6). This result suggests that the presence of 1,2-GG intrastrand crosslink of cisplatin interferes with top1 recognition of its DNA binding site and formation of top1–DNA cleavable complex.

In order to discriminate whether the cleavage activity of top1 is inhibited by distortion of the DNA structure caused by formation of the 1,2-GG intrastrand crosslink *per se* or by chemical modification of the guanine bases

adjacent to the cleavage site by the platinum complex, we also prepared substrates containing single, site-specific adduct of monodentate [PtCl(dien)]Cl which preferentially forms monofunctional adducts at N7 of guanine residues. This type of adducts has been shown to cause markedly less extensive distortions in DNA structure compared to 1,2-intrastrand crosslink of bidentate cisplatin (24,36). The upper (scissile) strand of the substrate contains two consecutive guanines, which are all preferential binding sites of [PtCl(dien)]Cl (37). To ensure that exclusively only one guanine at the +1 or +2 position of the 30-bp duplex (Figure 6A) is modified by [PtCl(dien)]Cl, either guanine at +1 or +2 position was replaced by 7-deaza-guanine (7-deazaG). The 7-deazaG is an isosteric analog of native guanine, in which the aromatic N7 atom is replaced with a C-H and which accurately mimics the properties of the natural base. As the guanine N7 is the site where platinum complexes preferentially react in DNA, 7-deazaG is incapable of forming platinum adducts (38). Thus, 7-deazaG makes it possible to prepare a single monofunctional adduct of [PtCl(dien)]Cl at the guanine residue at either +1 or +2 position. The results shown in Figure 6C, lanes 3, 5, 9, 11) demonstrate that replacement of guanines by 7-deaza-dG in nonmodified substrate has no effect on the cleavage activity of top1. On the other hand, modification of guanine at either +1 or +2 position by [PtCl(dien)]Cl completely inhibits cleavage of the



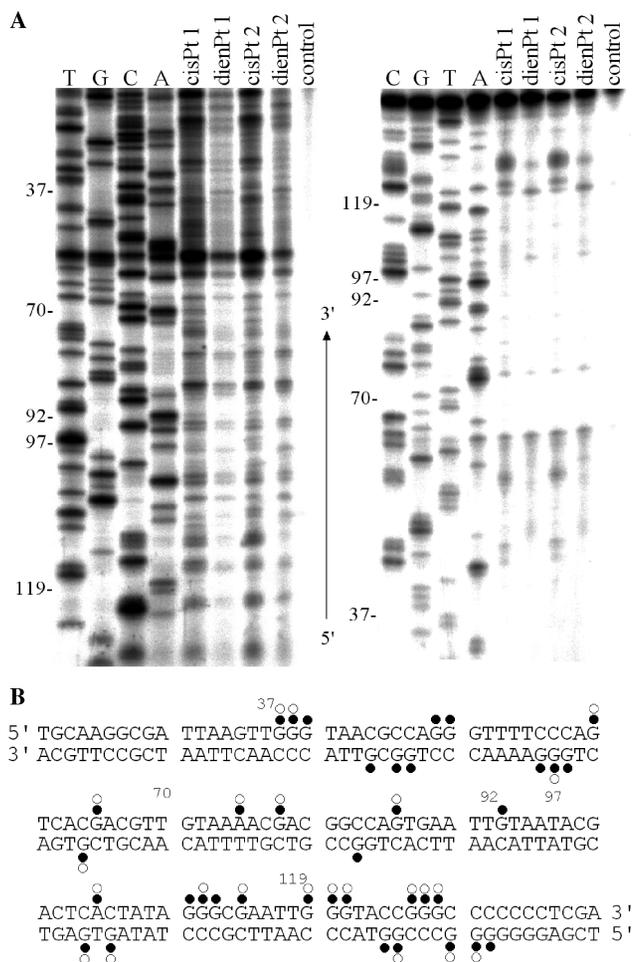
**Figure 4.** The dependence of the DNA cleavage of the 161-bp PvuII/HindIII fragment of pBluescript SK(-) phagemid mediated by top1 in the presence of CPT on the level of DNA modification by cisplatin (A) and [PtCl(dien)]Cl (B). The relative cleavage was calculated as a ratio of the intensities of the bands corresponding to the five main cleavage sites (Figure 3) induced by top1 in unplatinated and platinated DNA in the presence of CPT after removal of the platinum adducts by treatment with NaCN.

substrate by top1 and no cleavage is observed even in the presence of CPT. Thus, these results (Figure 6C) are identical to those obtained with cisplatin (Figure 6B) and demonstrate that top1 is very sensitive to the modification of bases in a close proximity of its cleavage site by platinum complexes.

## DISCUSSION

Chemical agents able to interfere with DNA topoisomerases are widespread in nature, and some of them have clinical efficacy as antitumor or antibacterial drugs. Drugs which have as a target DNA topoisomerases can be divided into two categories: poisons and suppressors (39). Classical topoisomerase 'poisons' stimulate cleavage in a sequence-selective manner, yielding drug-specific cleavage intensity pattern. Top1 'suppressors' inhibit the ability of top1 to cleave the phosphodiester backbone of supercoiled DNA by preventing or reversing top1-DNA complex formation (40). The suppressors may modify DNA chemically or even alter DNA conformation, preventing top1 binding to the top1 cleavage sites (41,42).

Our results indicate that the presence of platinum adducts in the substrate DNA inhibits top1 action in a



**Figure 5.** (A) Autoradiograms of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by VentR(exo-) DNA polymerase on the 161-bp PvuII/HindIII fragment of pBluescript SK(-) phagemid and subsequently modified by cisplatin or [PtCl(dien)]Cl. The gels contained the linear amplification products of control, nonplatinated DNA, and DNA treated with cisplatin or [PtCl(dien)]Cl at  $r_b = 0.005$  or  $0.008$ . Lanes are as follows: (control) unmodified template; lanes, cisPt1 and dienPt1, DNA modified by cisplatin or [PtCl(dien)]Cl, respectively, at  $r_b = 0.005$ ; lanes, cisPt2 and dienPt2, DNA modified by cisplatin or [PtCl(dien)]Cl, respectively, at  $r_b = 0.008$ ; lanes C, G, T, A, chain-terminated marker DNAs (note that these dideoxy sequencing lanes give the sequence complementary to the template strand). The numbers correspond to the nucleotide sequence numbering of part B. Left panel, mapping of the Pt adducts formed on the top strand of the 161-bp fragment; right panel, mapping of the Pt adducts formed on the bottom strand of the 161-bp fragment. (B) Schematic diagram showing a portion of the sequence used to monitor inhibition of DNA synthesis on the template containing adducts of cisplatin or [PtCl(dien)]Cl. Solid circles indicate major stop signals from part A, lane cisPt2, open circles indicate major stop signals from part A, lane dienPt2. The numbering of the nucleotides in this scheme corresponds to the cleavage sites of top1 (Figure 4). Nucleotides 37, 70, 92, 97 and 119 correspond respectively to nucleotides 569, 602, 624, 629 and 741 on the pBluescript SK(-) phagemid nucleotide sequence map.

manner dependent on the level of DNA platination. As determined by DNA relaxation assay, the yield of top1 catalysis decreases as a consequence of global modification of plasmid by antitumor cisplatin considerably more than due to modification by [PtCl(dien)]Cl (Figure 2).



the [PtCl(dien)]Cl adducts is slightly less pronounced. This observation may be interpreted to mean that severe conformational alterations induced in DNA by the 1,2-GG intrastrand crosslink of cisplatin, which is the major adduct formed in DNA by this metallodrug, are not implicitly critical for suppression of DNA cleavage by top1 in the presence of CPT. Also interestingly, DNA cleavage at some sequences containing only one guanine residue in the immediate proximity of the cleavage site on the scissile strand (at the 70 and 92 positions, Figure 4) is not suppressed or is suppressed only slightly if the fragment was globally modified by cisplatin. The observation that DNA cleavage at the site 70 is almost unaffected by the global modification of the fragment by cisplatin or [PtCl(dien)]Cl is apparently associated with the fact that the single G immediately downstream of the cleavage site 70 was not metallated by these complexes (Figure 5). DNA cleavage was suppressed at the site 92 only slightly even when the neighboring single G at the 93 site was metallated by cisplatin. Similarly, DNA cleavage was also suppressed only slightly at the site 97 when the neighboring single A at the 98 site was metallated by [PtCl(dien)]Cl. Thus, rather a multiple metallation of the short nucleotide sequences on the scissile strand, immediately downstream of the cleavage site and/or platination of the residues at the +2 and/or +3 position appears to impede the cleavage by top1 in the presence of CPT.

In order to shed light on the effect of platination of guanine residues at the +1 and +2 positions on the scissile strand, we also examined cleavage by top1 in the absence or presence of CPT of the 30-bp duplex (Figure 6A), which contains a single high-affinity top1 cleavage site on the scissile strand [between thymine (-1) and two guanines (+1 and +2) (Figure 6A)]. The results of these experiments (Figure 6B and C) confirm that even a single platination of the guanine residue by [PtCl(dien)]Cl at the +2 position effectively suppresses DNA cleavage by top1 in both the presence or absence of CPT. This result further supports the view that platination of the guanine residue not immediately downstream of the cleavage site at which top1 normally cleaves is important in the mechanism of suppression of the catalytic activity of top1.

The mechanism of enhanced cleavage of DNA in the presence of CPT involves stabilization of the normally transient cleavable DNA-top1 complex and formation of an enzyme-drug-DNA ternary cleavable complex (49). It has been suggested (50) that this ternary complex is stabilized not only by interactions between CPT and top1, but also by interaction between intercalated CPT and a flipped base at the +1 position. Therefore, it seems reasonable to speculate that a multiple platination of bases on the scissile strand, immediately downstream of the cleavage site and/or platination of the guanine residues at the +2 and/or +3 position may impede interaction between intercalated CPT and a flipped base at the +1 position by hampering either CPT intercalation and/or flipping the guanine base at the +1 position. Other experiments are needed to support this hypothesis.

An intriguing finding is that DNA cleavage by top1 in the presence of CPT at the sites corresponding to the

92 and 97 positions was notably enhanced in particular at lower levels of global modification of the 161-bp fragment by [PtCl(dien)]Cl or cisplatin, respectively (Figure 4). This enhancement was only observed if no residues in the immediate proximity of the cleavage sites 92 or 97 were platinated. Hence, a plausible explanation of this enhancement of DNA cleavage by top1 may consist in the inability of top1 to bind to its cleavage sites 37 and 119 due to multiple metallation by cisplatin or [PtCl(dien)]Cl immediately downstream of these cleavage sites. Thus, more molecules of top1 could be available for cleavage at the sites where top1 normally cleaves and where platination does not interfere in the immediate vicinity of these cleavage sites. In other words, enhancement of DNA cleavage by top1 in the presence of CPT at the sites corresponding to the 92 and 97 positions (Figure 4) was not due to platinum-induced structural change in DNA, but rather due to displacement of the top1/CPT complex from platinum-DNA binding sites, thus increasing its concentration elsewhere. Similar phenomenon was observed earlier in DNaseI footprinting experiments (51,52). This hypothesis is also corroborated by the fact that the reaction, in which top1-mediated cleavage of the 161-bp DNA fragment was examined (Figure 3), contained one molecule of top1 per more than 200 top1 cleavage sites in the sample of the 161-bp fragment. On the other hand, it should be pointed out that this 'displacement' mechanism may not necessarily act in an *in vivo* situation when the level of DNA platination in cells treated with platinum complexes is pronouncedly lower.

In this respect, an earlier study of Schellens and coworkers (18) dealing with the effect of DNA adducts of cisplatin on poisoning of top1 by CPT deserves further discussion. This study has demonstrated that single, site-specific 1,3-GTG intrastrand crosslink of cisplatin formed in the 24-bp duplex produces two cleavage sites of top1 on the platinated strand in the presence of CPT. One cleavage site, between C and T residues of the platinated strand, was separated by one nucleotide downstream of the 3' guanine involved in the 1,3-GTG intrastrand crosslink. DNA cleavage was enhanced at this site as a consequence of the presence of the 1,3-intrastrand crosslink. The other top1 cleavage site (not observed in the unplatinated duplex) occurred in the same strand between G and C residues in the immediate proximity of the 3' G residue of the crosslink. These results have been interpreted to mean that the 1,3-GTG intrastrand crosslink of cisplatin acts as a top1 poison (18). Nevertheless, the 1,3-GTG intrastrand crosslink of cisplatin examined in the work of Schellens and coworkers (18) only represents a minor DNA adduct of cisplatin which distorts DNA in a way distinctly different from that by which DNA is distorted by the major adducts of cisplatin, such as 1,2-GG or AG intrastrand crosslink (4,53). In addition, the sequence of the 24-bp oligonucleotide used in their study contained no canonical (preferential) cleavage site of top1 so that a direct comparison with the results of the present study cannot be straightforward.

Top1 breaks double-helical DNA at sites that occur, on the average, more frequently than 1 per 10 bases (44).

Even if one considers only the strong sites, which occur on the average every 30 to 35 bases on one strand, a random sequence of duplex DNA will contain a site every 1 to 2 turns of the helix. Thus, it cannot be excluded that at the levels of platination of DNA in tumor cells of patients treated with antitumor cisplatin this metaldrug may also bind at or close to the DNA cleavage sites of top1. It might be so also because canonical cleavage sites of top1 contain a number of high affinity binding sites for cisplatin and other antitumor platinum drugs. It seems therefore quite reasonable to suggest that mechanisms underlying antitumor effects of cisplatin and perhaps of other platinum drugs may also involve effects of these metaldrugs on top1 catalytic activity.

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