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Interactions of DNA with a New Platinum(IV) Azide Dipyridine Complex Activated by UVA and Visible Light. Relationship to Toxicity in Tumor Cells

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Abstract

The Pt$^{IV}$ diazido complex trans,trans,trans-$\text{[Pt(N}_3\text{)}_2\text{(OH)}_2\text{(pyridine)}_2\text{]}$ (I) is unreactive in the dark but is cytotoxic when photoactivated by UVA and visible light. We have shown that I when photoactivated accumulates in tumor cells and binds strongly to nuclear DNA under conditions in which it is toxic to tumor cells. The nature of the DNA adducts, including conformational alterations, induced by photoactivated I are distinctly different from those produced in DNA by conventional cisplatin or transplatin. In addition, the observation that major DNA adducts of photoactivated I are able to efficiently stall RNA polymerase II more efficiently than cisplatin suggests that transcription inhibition may contribute to the cytotoxicity levels observed for photoactivated I. Hence, DNA adducts of I could trigger a number of downstream cellular effects different from those triggered in cancer cells by DNA adducts of cisplatin. This might lead to the therapeutic effects that could radically improve chemotherapy by platinum complexes. The findings of the present work help to explain the different cytotoxic effects of photoactivated I and conventional cisplatin and thereby provide new insights into mechanisms associated with the antitumor effects of platinum complexes photoactivated by UVA and visible light.

KEYWORDS: Anticancer platinum; cytotoxicity; photoactivation; DNA damage; RNA polymerase II
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INTRODUCTION

It has been demonstrated that Pt$^{IV}$ diazido complexes which are stable and non-cytotoxic in the dark exhibit significant toxicity in cancer cells upon irradiation with short wavelengths (365 nm) (1). Their activation with UVA irradiation to produce cytotoxic and reactive Pt$^{II}$ analogues does not require oxygen (1, 2), an advantage over conventional photosensitizers currently used in photodynamic therapy (PDT), such as those based on tetrapyrrrole derivatives. In addition, selective photoactivation of platinum complexes in cancer cells may help to overcome limitations connected with toxic side effects of antitumor platinum drugs currently used in the clinic. The first photoactivatable Pt$^{IV}$ complexes exhibiting toxicity in tumor cells could be photoactivated only upon irradiation with wavelengths in UVA region (1), which is not optimal for clinical applications. Recently, the Pt$^{IV}$ diazido complex $trans,trans,trans$-[Pt(N$_3$)$_2$(OH)$_2$(pyridine)$_2$] (1) was synthesized which is both highly soluble and stable in aqueous solution. It can be photoactivated over a range of wavelengths and is toxic towards cancer cells using low doses of UVA radiation and, crucially, also with visible light (3). The presence of planar pyridine ligands in 1, which appear to remain strongly bound to platinum, even after photoactivation, has a critical effect not only on the photoactivation pathways, but also on the biological activity. Modification of clinically-ineffective transplatin ($trans$-[PtCl$_2$(NH$_3$)$_2$]) by inclusion of planar ligands considerably changes the binding mode of the $trans$-Pt$^{II}$ species with DNA, the major pharmacological target of antitumor platinum complexes (4-15). These transplatin analogues exhibited promising toxic effects in various human tumor cell lines including those resistant to conventional cisplatin. Thus, photoactivated 1 can be coclustered with other platinum-pyridines which are not cross-resistant and which exhibit different mechanisms of action from platinum-based drugs already on the market such as cisplatin, carboplatin and oxaliplatin.

Cisplatin and its analogues carboplatin and oxaliplatin bind to DNA preferentially at the N7 position of guanine bases (16, 17), inhibiting replication (18, 19) and transcription (20, 21). The inhibition of these critical DNA-related processes triggers subsequent intracellular events that activate apoptotic and
necrotic pathways (22). Hence, similar to other antitumor platinum-pyridine complexes, the different mechanism of action of photoactivated 1 may also derive from its unique mode of binding to DNA.

To assess the importance of the binding of photoactivated 1 to DNA for the efficacy of its anticancer action, we describe here an investigation of DNA binding of photoactivated 1 in tumor cells treated with this metallodrug under conditions where it is non-toxic towards these cells (in the absence of irradiation). In addition, the fact that photoactivated 1 binds to DNA in cells prompted us to investigate the modifications induced in DNA in vitro (in cell-free media), including conformational alterations of DNA and the processing of these modifications by RNA polymerase II. We believe that a deep understanding of the reactions leading to bifunctional DNA cross-link (CL) formation by photoactivated 1 may provide guidance in future efforts to optimize the rational design of photoactivatable anticancer metallodrugs.
EXPERIMENTAL PROCEDURES

Chemicals. *Trans,trans,trans*-\([\text{Pt(N}_3)_2(\text{OH})_2(\text{py}_2)]\) (I) was synthesized and characterized by the method described previously (3). *Cis*- and *trans*-\([\text{Pt(Cl}_2](\text{NH}_3)_2\] (cisplatin and transplatin) were obtained from Sigma (Prague, Czech Republic) (purity was 99.9 %). Chlorodidiethylenetriamineplatinum(II) chloride (\([\text{PtCl(dien)}]\)Cl) was a generous gift of Prof. G. Natili from the University of Bari. The stock solutions of platinum complexes were prepared in \(\text{H}_2\text{O}\), their concentrations determined by flameless atomic absorption spectroscopy (FAAS), and stored in the dark. Calf thymus (CT) DNA (42% G + C, mean molecular mass ca. 20 000 kDa) was prepared as previously described (23, 24). Plasmids pUC19 [2686 base pairs (bp)], pSP73 (2464 bp) and pSP73KB (2455 bp) were isolated according to standard procedures. The Klenow fragment from DNA polymerase I, restriction endonucleases *EcoRI* and *XbaI*, plasmid pCMV-GLuc (5764 bp), T4 DNA ligase and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). HeLaScribe® Nuclear Extract *in vitro* Transcription system kit, T7 and SP6 RNA polymerases and RNasin ribonuclease inhibitor were purchased from Promega (Mannheim, Germany). Ribonucleotide triphosphates were from Roche Diagnostics, GmbH (Mannheim, Germany). Sephadex G-50 (Coarse) was from Sigma-Aldrich (Prague, Czech Republic). Agarose and ethidium bromide (EtBr) were from Serva Electrophoresis GmbH (Heidelberg, Germany). TbCl\(_3\)·6 \(\text{H}_2\text{O}\) was from Fluka Chemie AG. Acrylamide, bis(acrylamide), dithiothreitol (DTT) and thiourea (TU) were from Merck (Darmstadt, Germany). The radioactive products were from MP Biomedicals, LLC (Irvine, CA).

Irradiation. The cells and DNA samples in cell-free media were irradiated using the LZC-4V illuminator (photoreactor) (Luzchem, Canada) with temperature controller and with UVA tubes (4.3 mW cm\(^{-2}\); \(\lambda_{\text{max}} = 365\) nm). DNA samples in cell-free media were also irradiated using LUXEON Star/O source (Light Emitting Diode) (Quadica Developments Inc., Brantford, Ontario, Canada) with optic that allows to aim the light source onto the sample (65 mW cm\(^{-2}\), \(\lambda_{\text{max}} = 458\) nm).

DNA Platination in Cells Exposed to Photoactivated I. The human ovarian carcinoma cisplatin sensitive A2780 cells were kindly supplied by Prof. B. Keppler from the University of Vienna (Austria).
A2780 cells in 10 cm dishes were treated without or with Pt complexes at the concentration of 24 µM
for 1 h in Earle's Balanced Salt Solution (EBSS). After this time, the cells were further kept in the dark
or irradiated with UVA for 1 h. Cells were then washed and incubated in the drug-free medium for
additional 24 h. At the end of incubation, the floating cells were collected and attached cells were
harvested by trypsinization. Total cells (floating + attached) were washed twice in PBS (4 °C) and lysed
in DNAzol (DNAzol® genomic DNA isolation reagent, MRC) supplemented with RNase A (100 µg
mL⁻¹). The genomic DNA was precipitated from the lysate with ethanol, dried and resuspended in water.
The DNA content in each sample was determined by UV spectrophotometry. To avoid interference from
high DNA concentrations on FAAS detection of platinum in the samples, the DNA samples were
digested in the presence of hydrochloric acid (11 M) using an high pressure microwave mineralization
system (MARS5, CEM). Experiments were performed in triplicate and the values are the means ±SD.

**Platination of DNA in Cell-free Media.** If not stated otherwise, CT DNA was mixed with 1 in
NaClO₄ (10 mM) and immediately irradiated (UVA, λₘₐₓ = 365 nm or visible light, λₘₐₓ = 458 nm) for
indicated time and then kept at 37 °C in the dark. The rᵢ value was 0.05 (rᵢ is defined as the molar ratio of
free platinum complex to nucleotide phosphates at the onset of incubation with DNA). Aliquots were
removed at various time intervals, quickly filtered using a Sephadex G-50 column to remove free
(unbound) Pt. The Pt content in these DNA samples (rᵇ, defined as the number of the molecules of
platinum complex coordinated per nucleotide residue) was determined by FAAS.

**DNA Interstrand Cross-linking.** Plasmid DNA pSP73KB was linearized by EcoRI and 3’-end
labeled by means of Klenow fragment of DNA polymerase I and [α-³²P]dATP. Complex 1 at varying
concentrations (rᵢ values) was mixed with 300 ng of linear pSP73KB in 10 mM NaClO₄ and irradiated
(λₘₐₓ = 365 nm or λₘₐₓ = 458 nm) for 1 h. The mixture was then incubated in the dark for 23 h. The
amount of interstrand CLs was analyzed by electrophoresis under denaturing conditions on alkaline
agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to
single strands of DNA and interstrand cross-linked duplex were quantified. The number of interstrand
cross-links (CLs) per adduct (%ICL/Pt) was calculated as %ICL/Pt = XL/4910 × rᵦ (pSP73KB plasmid
contained 4910 nucleotide residues). XL is the number of interstrand CLs per molecule of the linearized DNA duplex which was ascertained assuming Poisson distribution of the interstrand CLs as XL = -ln A, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

**Unwinding of Negatively Supercoiled DNA.** Unwinding of closed circular supercoiled pUC19 plasmid DNA was assayed by an agarose gel mobility assay (25). The unwinding angle Φ, induced per Pt–DNA adduct was calculated by the determination of the r_b value at which the complete transformation of the supercoiled to relaxed form of the plasmid was attained. Samples of pUC19 plasmid were mixed with 1 in NaClO_4 (10 mM) and irradiated (λ_max = 365 nm or λ_max = 458 nm) for 1 h. The samples were then incubated at 37 °C in the dark. After 23 h, all samples were redissolved in the Tris-acetate/EDTA (TAE) buffer and subjected to electrophoresis on 1% agarose gels running at room temperature with TAE buffer and the voltage set at 35 V. The gels were stained with EtBr, followed by photography with a transilluminator.

**Transcription Mapping of DNA Adducts In Vitro.** Transcription of the (NdeI/HpaI) restriction fragment of pSP73KB DNA treated with 1 under irradiation conditions (λ_max = 365 nm or λ_max = 458 nm for 1 h and subsequently 23 h in the dark) with DNA-dependent T7 RNA polymerase and electrophoretic analysis of transcripts were performed according to the protocols recommended by manufacturer (Promega Protocols and Applications, 43-46, 1989/90) and described in detail previously (26, 27). The concentration of DNA used in this assay was 3.9 x 10^{-5} M (relative to the monomeric nucleotide content). The r_i values for platination reactions were chosen so as to obtain an r_b value of 0.01. The drug not bound to DNA was removed by ethanol precipitation.

**Fluorescence Measurements.** These measurements were performed on a Shimadzu RF 40 spectrofluorophotometer using a 1-cm quartz cell. Fluorescence measurements of CT DNA modified by platinum complexes in the presence of EtBr were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25 °C in NaCl (0.4 M) to avoid secondary binding of EtBr to DNA (28, 29). The concentrations were 0.01 mg mL^{-1} for DNA and 0.04 mg mL^{-1} for EtBr, which corresponded to the saturation of all sites of EtBr
in DNA (29). Terbium fluorescence measurements were performed by adding TbCl₃ to modified or control DNA (8 µg mL⁻¹) at a final concentration equivalent twice the monomeric nucleotide content. The fluorescence intensity was measured after equilibration for 60 min at 25°C in the dark. The excitation and emission wavelengths were 290 nm and 546 nm, respectively. Other details of these measurements can be found in papers published earlier (30-32).

DNA Transcription by RNA Polymerase II In Vitro. The in vitro transcription assay was performed using HeLaScribe® Nuclear Extract in vitro Transcription system kit. All components for in vitro transcription from a CMV promoter of plasmid pCMV-GLuc are contained in this system. Complex I at varying concentrations was incubated for 1 h under irradiation conditions (λ_max = 365 nm or λ_max = 458 nm for 1 h and subsequently 23 h in the dark) with 100 ng of pCMV-GLuc DNA linearized by XbaI. The samples were then kept at 37 °C in the dark for 23 h. After modification, the excess of drug was removed by ethanol precipitation. HeLa nuclear extract supplied with the kit was used along with the protocol for in vitro transcription assay recommended by the manufacturer with small modifications. Platinated or nonplatinated linearized pCMV-GLuc DNA was incubated in the transcription buffer supplemented with MgCl₂ (4 mM), rATP (0.4 mM), rCTP (0.4 mM), rGTP (0.4 mM), UTP (16 µM), 10 mCi [α-³²P]UTP (3000 Ci/mmol), RNase inhibitor (20 U) and nuclear extract (8 U) in a final reaction volume of 25 mL at 30 °C. After 60 min, the reaction was terminated by the addition of 175 mL HeLa Extract Stop Solution and phenol-chloroform extraction followed. Then the transcripts were precipitated by ethanol and the pellet was washed, dried and resuspended in a loading buffer containing formamide (90%), EDTA (10 mM), xylene cyanol (0.1%) and bromphenol blue (0.1%). The samples were separated by electrophoresis on a 6% denaturing polyacrylamide (PAA) gel. The gels were then visualized and the radioactivity associated with bands was quantitated.

Single-lesion Substrates for DNA Transcription by T7 and SP6 RNA Polymerases In Vitro. The 69-bp templates nonmodified or containing a central, single 1,3-GTG intrastrand CL of photoactivated 1 or 1,2-GG intrastrand CL of cisplatin were assembled from four oligodeoxyribonucleotide strands as illustrated in Figure 9A. The central oligonucleotide [23 nucleotides (nt)] containing GTG sequence was
mixed with 1 in their equimolar concentrations, irradiated by UVA for 30 min and subsequently incubated in the dark for 24 h. The platinated oligonucleotide was purified by using ionic-exchange HPLC. A product was collected, dialysed against double-distilled water and the unique platinations at the guanine sites were verified by FAAS and DMS footprinting of platinum bound to DNA, as already described (33). Cisplatin-modified oligonucleotide containing central GG sequence was prepared by the same procedure except for the omission of the irradiation step. The central oligonucleotides were then annealed with their bottom strands (69-mers) and two oligonucleotides (two marginal arms, each 23 nt long) were ligated (one to each side) to these duplexes by T4 DNA ligase. Full-length substrates (nonmodified, containing the 1,3-GTG intrastrand CL of photoactivated 1 or the 1,2-GG intrastrand CL of cisplatin) were separated from unligated products on a denaturing 12 % PAA gel, purified by electroelution, reannealed, and stored in NaClO₄ (0.01M).

**T7 and SP6 RNA Polymerases Reaction on Single-lesion Templates.** T7 and SP6 polymerization reactions were performed using Riboprobe® In Vitro Transcription Systems Protocol (Promega, Mannheim, Germany) according the recommended protocol. Briefly, unplatinated or single-lesion-containing 69 bp templates (2 pmol) were incubated at 37 °C in 20 µL of buffer containing Tris-HCl (40 mM, pH 7.9), NaCl (10 mM), MgCl₂ (6 mM), DTT (10 mM), spermidine (2 mM), Tween®-20 (0.05%), 0.5 mM each of rATP, rCTP and rUTP, rGTP (0.125 mM) and 0.5µCi [α-³²P]rGTP. The reactions were initiated by the addition of 15 units of T7 polymerase. After 1 h of incubation, reaction mixtures were precipitated by ethanol and resolved by electrophoresis on 12% PAA/8M urea gel. RNA sequencing lanes were generated by adding of 3´-dCTP or 3´-dATP into the reactions containing nonplatinated 69-bp constructs as a template. The reaction with SP6 polymerase was performed as described for T7 polymerase (vide supra) except that the concentration of rGTP was 0.5 mM, rUTP 0.125 mM and 0.5 µCi [α-³²P]rUTP was used.

**Other Physical Methods.** Absorption spectra were obtained on a Beckman 7400 DU spectrophotometer equipped with a thermostated cell holder. The FAAS measurements were carried out on a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube
atomizer. The gels were visualized on a BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivity associated with bands was quantified with the AIDA image analyzer software (Raytest, Germany).
RESULTS

DNA-bound Platinum in Cells Exposed to Photoactivated 1. Distortions of DNA structure by metallodrugs often correlate with anticancer activity (34). Hence, it is of great importance to understand in detail the DNA binding properties of the photoactivatable Pt^IV complex 1 and the possible relationship with cytotoxicity in tumor cell lines.

We examined platinated DNA isolated from ovarian carcinoma A2780 cells after exposure to 1 under irradiation conditions (UVA) or in the dark. Importantly, 1 was potently phototoxic towards a number of human tumor cell lines including ovarian A2780 cancer cells (3). After the treatment was completed (see Experimental Procedures), DNA was isolated and the Pt content was determined by FAAS. Measurements of DNA-bound platinum after 1 h of 24 µM drug exposure under irradiation conditions (365 nm, 4.3 mW cm^-2) revealed that the amount of platination by 1 was 700±84 fmol Pt/µg DNA. This amount was approximately 16-fold higher compared to that determined after 1 h treatment with the same dose of cisplatin in dark (43±8 fmol Pt/µg DNA). Importantly, no Pt bound to DNA was observed if the cells were treated with 1 in the dark. These results confirm that the binding of 1 to DNA in tumor cells correlates with the conditions under which it exhibits cytotoxicity – binding to DNA and cytotoxicity with irradiation, and no binding to DNA or cytotoxicity in the absence of irradiation.

DNA Binding of Photoactivated 1 in Cell-free Media. Kinetics of Binding to Calf Thymus DNA. The first experiments were aimed at quantifying the binding of 1 to mammalian DNA in cell-free media. Two samples of double-helical CT DNA (32 µg mL^-1) were incubated with 1 at an r_i value of 0.05 in NaClO_4 (10 mM) at 37 °C. The first sample was irradiated with UVA (365 nm, 4.3 mW cm^-2) or visible (458 nm, 65 mW cm^-2) light immediately after addition of 1; the other sample was kept in the dark. Aliquots of both samples were withdrawn at various time intervals; free, unbound platinum was removed by gel filtration through Sephadex G-50 coarse column and DNA was assayed for platinum content by FAAS. Only a very small amount (<5%) of platinum bound to DNA was found in the sample which was kept in the dark, even after a long time of incubation (24 h; Figure 2A). In contrast, the
amount of platinum bound to DNA in irradiated samples increased with time. Under continuous UVA irradiation, 50% of Pt was bound after 70 min ($t_{50\%}$) and the plateau of the platination was reached after 7.5 h continuous irradiation, when nearly 100% of platinum present in the sample was bound to DNA (Figure 2A). Similarly, 50% of Pt was bound after 106 min ($t_{50\%}$) and the plateau of the platination reaction was reached after 7.5 h continuous irradiation by visible light, when nearly 70% of platinum present in the sample was bound to DNA (Figure 2C). Interestingly, when I was first pre-irradiated by UVA for 2 h (in the absence of DNA) and only then added to DNA in the dark, the DNA binding rate was markedly slower and a maximum amount of platinum bound to DNA after 7.5 h was lower as well compared to the situation when I was continuously irradiated in the presence of DNA (Figure 2A).

Unfortunately, extended periods of irradiation in particular by UVA (7.5 h) caused severe DNA damage which made subsequent analysis of conformational properties of DNA modified by photoactivated I impossible. Therefore, other modes of irradiation were also tested (shown for UVA irradiation in Figure 2B). DNA was mixed with I at $r_i = 0.05$, samples were pre-irradiated for a preselected time (0.5, 1 or 2 h) and subsequently incubated in the dark. Aliquots were withdrawn and DNA was assayed for platinum content as described above. Figure 2B shows that the total amount of platinum bound to DNA after 24 h of post-irradiation incubation increases with the length of the pre-irradiation (in the presence of DNA). As mentioned above, when I was first irradiated for 2 h in the absence of DNA, then added to DNA and the sample was subsequently incubated in the dark, the amount of platinum bound to DNA was considerably lower than that when I was irradiated for 2 h in the presence of DNA and the sample was subsequently incubated in the dark (cf. Figures 2A and B). Qualitatively similar results were obtained if I or the mixtures of DNA with I were pre-irradiated with visible light, although yields of DNA binding reactions were lower compared to those when the mixtures were pre-irradiated by UVA.

Thus, these DNA binding experiments (Figure 2) clearly indicated that I was inactive and unable to bind DNA in the dark, but under irradiation by UVA or visible light it became active and strongly bound DNA.
The results of these DNA binding studies in cell-free media indicate that the rates of binding to
natural double-helical DNA, when the reaction mixture was irradiated by UVA or visible light, were
relatively high compared to the binding of non-irradiated conventional cisplatin or transplatin (35). The
binding experiments carried out here also indicate that the modification reactions result in the
irreversible coordination of photoactivated 1 to double-helical DNA, which also facilitates sample
analysis. Hence, it is possible to prepare samples of DNA modified by 1 photoactivated by UVA or
visible light at a preselected value of \( r_b \) (the number of molecules of the platinum complex bound per
nucleotide residue). The samples of DNA treated with photoactivated 1 and analyzed further by
biophysical or biochemical methods were prepared in NaClO\(_4\) (10 mM) at 37 °C. If not stated
otherwise, after 1h of the treatment of DNA with the complex under continuous irradiation by UVA or
visible light, the samples were incubated in the dark for an additional 23 h. After that, the samples were
precipitated in ethanol, dissolved in the medium necessary for a particular analysis and the \( r_b \) value in an
aliquot of this sample was checked by FAAS. In this way, the analyses described in the present paper
were performed in the absence of unbound (free) platinum complex if not stated otherwise.

**Characterization of DNA Adducts of Photoactivated 1 with Thiourea.** Cisplatin, transplatin, and
analogs of these bifunctional platinum compounds coordinate to DNA in a two-step process, forming
first monofunctional adducts preferentially at guanine residues; these monofunctional adducts
subsequently close to bifunctional lesions (35, 36). Considerable evidence suggests that
monofunctionally-bound transplatin and its analogs are labilized by thiourea, whereas bifunctional
adducts are resistant (37, 38). TU is used to labilize monofunctionally bound transplatin and its
analogs from DNA (37). The displacement of transplatin or its analogues is initiated by coordination
of TU trans to the nucleobase. Owing to the strong trans effect of sulfur, the nucleobase nitrogen–
platinum bond is weakened and thus becomes susceptible to further substitution reactions.
Consequently, transplatin or its analogues in monofunctional DNA adducts are effectively removed,
whereas bifunctional adducts of transplatin or its analogues are resistant to TU treatment (37).
The experiments aimed at the characterization of DNA adducts of photoactivated 1 were conducted employing TU as a probe for DNA monofunctional adducts formed by this trans-platinum compound. Double-stranded CT DNA was mixed with 1 at a drug-to-nucleotide ratio of \( r_i = 0.05 \) in NaClO₄ (10 mM), irradiated for 1 h by UVA or visible light and then incubated in the dark for an additional 23 h. During this period the platination reaction was stopped at various time intervals by addition of NaCl (final concentration 0.1 M) and immediate cooling to -20 °C or, in parallel experiments, by addition of TU (final concentration was 10 mM). The samples treated with TU were still incubated for an additional 10 min at 25 °C and then quickly cooled to -20 °C. The DNA samples were then filtered using Sephadex G50 coarse columns to remove low molecular mass fractions and the platinum content was determined by FAAS (Figure 3). The reaction of DNA with 1 photoactivated by UVA reached ~70% after 24 h, consistent with the results of DNA binding experiments (Figure 2B). TU displaced approximately 50% of already bound photoactivated complex 1 from DNA at early time intervals (1–5 h; Figure 3A). At longer incubation times (24 h), TU was slightly less efficient in removing 1 from DNA (~37% of total Pt adducts), which suggests that a considerable fraction of monofunctional adducts of 1 photoactivated by UVA had closed to bifunctional lesions already during the early time of incubation. Thus, after a reaction period of 24 h, 63% of 1 photoactivated by UVA bound to DNA had evolved to bifunctional lesions and therefore, was not displaced from double-stranded DNA by TU (Figure 3A), which implies that approximately 37% of total adducts remained monofunctional. A similar experiment was performed using visible light to irradiate the mixtures of CT DNA and 1 (Figure 3B). Under these conditions, similar results were obtained to those found for the mixtures irradiated by UVA; after 24 h of incubation, TU displaced 31% of total platinum adducts from DNA which implies that 69% of Pt adduct formed on DNA by 1 photoactivated by visible light had evolved into CLs.

**DNA Interstrand (Intramolecular) Cross-linking by Photoactivated 1.** Bifunctional platinum compounds, which coordinate base residues in DNA, form various types of interstrand and intrastrand CLs. Such CLs in the target DNA are important factors involved in the DNA damaging action of genotoxic agents. Therefore, we have quantitated the interstrand cross-linking efficiency of
photoactivated 1 in pSP73KB plasmid (2455 bp). This plasmid DNA was linearized by EcoRI (EcoRI
cuts only once within pSP73KB plasmid) and radioactively labeled on its 3′-end.

Plasmid DNA was incubated with 1 at varying concentrations, irradiated for 1 h by UVA or visible
light and incubated in the dark for an additional 23 h. The samples were then analyzed for interstrand
CLs by agarose gel electrophoresis under denaturing conditions (39). Upon electrophoresis, 3′-end
labeled strands of linearized pSP73KB plasmid containing no interstrand CLs migrate as a 2455-base
single strand, whereas the interstrand cross-linked strands migrate more slowly as a higher molecular
mass species (Figure 4). The intensity of the more slowly migrating DNA fraction increased with the
growing level of the platination. The radioactivity associated with the individual bands in each lane was
measured to obtain estimates of the fraction of noncross-linked or cross-linked DNA under each
condition. The frequency of interstrand CLs of photoactivated 1 was calculated as described earlier (38)
and was found to be 12 ± 2% (mean and standard deviation calculated from three independent
experiments) for DNA modified by 1 photoactivated by both UVA and visible light.

Unwinding of Negatively Supercoiled DNA by Adducts of Photoactivated 1. The binding of low-
molecular-mass compounds such as antitumor metallodrugs to closed circular DNA can cause
defformation and shift of the base pairs, which can lead to partial unfolding of the DNA. This process
lowers the superhelical density of plasmid DNA, which causes a decrease in the rate of migration
through an agarose gel. This fact makes it possible to observe and quantify the mean value of unwinding
per adduct. In the present study, we investigated the unwinding induced in negatively supercoiled
pUC19 plasmid treated with 1 photoactivated by UVA or visible light. The degree of supercoiling was
monitored using electrophoresis in native agarose gel. We investigated the effect of increasing amounts
of 1 photoactivated by UVA or visible light bound to a mixture of relaxed and supercoiled pUC19 DNA
on migration of these forms of pUC19 DNA in a native agarose gel (shown in Figure 5 for DNA
modified by 1 photoactivated by UVA radiation). The unwinding angle is given by \( \Phi = -18\sigma/r_b(c) \),
where \( \sigma \) is the superhelical density and \( r_b(c) \) is the value of \( r_b \) at which the supercoiled and nicked forms
comigrate (25). The DNA unwinding angle determined for DNA modified by 1 photoactivated by UVA
was 28±3°. An identical experiment carried out with plasmid treated with 1 photoactivated by visible light (not shown) yielded an unwinding angle 27±3°.

### Transcription Mapping of DNA Adducts of Photoactivated 1.

Cutting of pSP73KB DNA by NdeI and HpaI restriction endonucleases yielded a 212-bp fragment (a substantial part of its nucleotide sequence is shown in Figure 6B). This fragment contained T7 RNA polymerase promotor [in the upper strand close to its 3'-end (Figure 6B)]. The first experiments were carried out using this linear DNA fragment, randomly modified by transplatin or cisplatin in the dark and by 1 photoactivated by UVA or visible light at \( r_b = 0.01 \), for RNA synthesis by T7 RNA polymerase (Figure 6A, lanes transPt, cisPt, 1(UVA) and 1(VIS) respectively). RNA synthesis on the template modified by the platinum complexes yielded fragments of defined sizes (Figure 6A), which indicates that RNA synthesis on these templates was prematurely terminated. The sequence analysis revealed that the major bands resulting from termination of RNA synthesis by the adducts of transplatin and photoactivated 1 were similar, appearing mainly at G and C sites and to a considerably less extent also at adenine (A) sites (Figure 6B). Importantly, the sequence dependence of the inhibition of RNA synthesis by the adducts of transplatin and photoactivated 1 is considerably less regular than that by the adducts of cisplatin, indicating that 1 photoactivated by both UVA and visible light forms a greater variety of adducts with DNA and less regularly than cisplatin does.

### Characterization of DNA Adducts of Photoactivated 1 by EtBr Fluorescence.

EtBr as a fluorescent probe has been used to characterize perturbations induced in DNA by bifunctional adducts of several platinum compounds (13, 40). Double-helical CT DNA was first modified by cisplatin, transplatin, or 1 photoactivated by UVA or visible light. The levels of the modification corresponded to the values of \( r_b \) in the range between 0 and 0.08. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (shown in Figure 7A for DNA modified by 1 photoactivated by UVA). The decrease caused by the adducts of photoactivated 1 was markedly more pronounced than that induced by the DNA adducts of cisplatin or transplatin at equivalent \( r_b \) values. Identical experiment carried out with CT DNA treated with 1 photoactivated by visible light (not shown) yielded identical
results. It was verified that irradiation of CT DNA by UVA or visible light in the absence of \textit{I} for 5 h had no effect on EthBr fluorescence.

**Characterization of DNA Adducts of Photoactivated \textit{I} by Terbium Fluorescence.** Terbium ion (Tb$^{3+}$) fluorescence is used to investigate local perturbations of conformation induced in double-helical DNA by various physical or chemical agents, including cisplatin (31, 32). This assay is based on the observation that Tb$^{3+}$ fluorescence is strongly enhanced when the ion is bound to the phosphate moieties of G residues in distorted DNA regions (30-32). The modification of double-helical DNA by cisplatin has been shown to result in substantially increased fluorescence of the lanthanide cation, which binds to unplatinated G residues in distorted regions around the platination site (31, 32). In contrast, coordination of complexes such as clinically ineffective transplatin or monofunctional [PtCl(dien)]Cl to double-stranded DNA does not result in distortions of the helix structure that would affect terbium fluorescence (31, 32).

CT DNA was modified by cisplatin, transplatin, or \textit{I} photoactivated by UVA or visible light at $r_b$ values in the range of 0.02 - 0.08, the resulting samples were treated with TbCl$_3$ and the fluorescence measured as described in the section Materials and Methods ((shown in Figure 7B for DNA modified by \textit{I} photoactivated by UVA radiation)). In accord with previous results (31, 32), the modification by cisplatin resulted in a significant enhancement of terbium fluorescence, while the modifications by transplatin had only a negligible effect. The modification by photoactivated \textit{I} also resulted in an enhancement of the fluorescence, which was, however, markedly higher than that observed in the case of DNA modified by cisplatin (shown for Figure 7B). These results confirm that DNA adducts of photoactivated \textit{I} induce local conformational alterations in double-stranded DNA that are markedly more pronounced than those induced by cisplatin. This observation is in contrast to the modification of DNA by transplatin, which does not induce in DNA conformational distortion detectable by terbium fluorescence assay. Identical results were obtained when DNA was modified by \textit{I} photoactivated by visible light. It was verified that irradiation of CT DNA by UVA or visible light in the absence of \textit{I} for 5 h had no effect on terbium fluorescence.
Transcription of DNA Modified by Photoactivated 1 by RNA Polymerase II In Vitro. One of the key factors that is important for platinum drug-mediated cytotoxicity is the arrest of RNA synthesis by Pt-DNA CLs (21). Therefore, the ability of 1 to affect transcription activity of human RNA polymerase II (RNA pol II) was tested using HeLaScribe1 Nuclear Extract in vitro Transcription system kit. Using an analogous procedure as described earlier (41), the RNA pol II transcription template pCMV-Gluc either nonmodified or modified by 1 photoactivated by UVA or visible light was incubated with the HeLa nuclear extract supplied with this kit. This extract can support accurate transcription initiation by RNA pol II and exhibits both basal and regulated patterns of RNA transcription. This nuclear extract is also the source for a variety of transcription factors, DNA binding proteins and the enzymatic machinery involved in process of RNA synthesis (42). Specific transcription from the CMV promoter results in a runoff transcript 688 nucleotides in length. The generated full length transcripts can be subsequently detected by gel electrophoresis.

As seen in Figure 8A, in the absence of platination, a high level of full length transcript was observed. In contrast, a significant decrease in the amount of full-length transcript was observed as a result of increasing template modification by 1 photoactivated by both UVA and visible light (Figure 9A). The relative amount of full-length transcript generated from each reaction was quantitated and plotted as a function of the level of the template platination (r_b) (Figure 9B). The results obtained for DNA modified by cisplatin in the dark under identical conditions were also included for comparative purposes. Under the conditions employed, RNA pol II transcription was highly sensitive to even very low levels of platinum damage on the template DNA, the damage by photoactivated 1 being more effective in inhibiting RNA pol II transcription compared to cisplatin.

To investigate the possibility that RNA pol II catalytic activity was inhibited as a consequence of hijacking factors essential for RNA pol II initiation by DNA adducts of photoactivated 1 (41), the following competition experiments were performed. RNA pol II transcription of undamaged template pCMV-Gluc was examined in the presence of increasing levels of a second, exogenous pUC19 plasmid containing multiple lesions caused by either photoactivated 1 or cisplatin. As shown in Figure 8C, the
initial addition of control, undamaged exogenous plasmid resulted in an overall increase in the amount of transcript generated by pCMV-Gluc which only slightly increased upon the further addition of unplatinated plasmid. RNA pol II transcription of pCMV-Gluc template was significantly reduced by the addition of increasing amounts of cisplatin modified exogenous plasmid. In contrast, a negligible inhibition effect was seen if the transcription assay was performed in the presence of exogenous plasmid containing adducts of 1 photoactivated by UV or visible light (shown in Figure 8C for DNA modified by 1 photoactivated by UVA).

**Effect of the Single, Site-specific DNA Lesion of Photoactivated 1 on RNA Synthesis.** The previous results have shown that enhanced effects of photoactivated 1 on the inhibition of RNA polymerization does not result from hijacking of transcription factors that are indispensable for the transcription process. It is possible that platinum lesions formed by photoactivated 1 represent a stronger block for RNA polymerase than cisplatin adducts do. Therefore, to investigate the effect of a single lesion of 1 on the synthesis of RNA by RNA polymerase, 69 bp-long deoxyribo-oligonucleotide duplexes were constructed so that they contained a single lesion of photoactivated 1 or cisplatin in the top strand, approximately 20 or 16 bp downstream of the start site for T7 or SP6 polymerases, respectively (Figure 9A). The constructs used in these studies contained either the 1,2-GG intrastrand cross-link of cisplatin or the 1,3-GTG intrastrand CL of photoactivated 1, which is the major adduct formed in DNA by this photoactivated complex (*vide supra*). The presence of the drug lesions and the purity of the transcription templates were confirmed by gel electrophoresis, FAAS and DMS footprinting (33).

Figure 9B shows that the GTG construct control template is efficiently transcribed by T7 RNA polymerase to yield high levels of full length 54 nt transcript. In contrast, transcription of the template containing a single 1,3-GTG intrastrand CL by T7 RNA polymerase (which transcribed the top, platinated strand) resulted in a high level of shorter length transcripts arising from blockage of the T7 RNA polymerase by this lesion. Importantly, even after 1 h of reaction, no full-length transcript was observed. Sequence analysis revealed that the T7 RNA polymerase was blocked at sites both one base
prior to the adduct site and at the first platinated guanine (Figure 9B). Transcription by T7 RNA polymerase from the unplatinated GG construct templates yielded similar results to those of the unplatinated GTG template (Figure 9B). The 1,2-GG intrastrand CL of cisplatin was also a block to the progression to the T7 RNA polymerase, displaying a blockage pattern different from that of the GTG adduct of photoactivated 1. T7 RNA polymerase was blocked mainly at the sites of the adducts (one half prior to the first and second platinated guanine). In this case, the full length product was also observed, although the intensity of this fraction was markedly reduced compared to the unmodified template. From the intensity of the T7 RNA polymerase blockage sites relative to the intensity of the full-length transcript, it can be calculated (taking into account the relative number of radiolabeled nucleotides incorporated into the RNA) that there were 73% and 100% blockages by the major adducts formed by cisplatin and photoactivated 1, respectively.

The effect of the GTG-adduct formed by photoactivated 1 (in the top strand) on transcription of the bottom, unplatinated strand was also tested using the same constructs. SP6 RNA polymerase was employed which transcribed the bottom, unplatinated strand of the constructs (Figure 9B, right panel). The presence of adducts of both cisplatin and photoactivated 1 in the top strand of the construct had no effect on the ability of SP6 RNA polymerase to transcribe the bottom, unplatinated strand and synthesize full length products.
DISCUSSION

The cytotoxic effects of antitumor platinum drugs might arise from a number of mechanisms, including tumor cell accumulation, protein interactions, DNA modifications and their cellular processing (34, 43, 44). To support the view that DNA is a potential target for photoactivated 1, platinum levels on nuclear DNA were determined after exposure of A2780 tumor cells to 1 photoactivated by UVA. Measurements of DNA-bound platinum after exposure to the photoactivatable platinum drug under irradiation revealed that the amount of platination by 1 was markedly (~16-fold) higher compared to that determined after the treatment with the same dose of cisplatin in dark for the same time. Importantly, no Pt bound to DNA was observed if the cells were treated with 1 in the dark. These results confirm that 1 when photoactivated accumulates in tumor cells, penetrates the nucleus and binds strongly to nuclear DNA under conditions in which it exhibits cytotoxicity. This suggests that penetrating the nucleus and binding to nuclear DNA may provide an important contribution to the mechanism of cytotoxicity of photoactivated 1. DNA may therefore be a potential target for this cytotoxic photoactivatable platinum complex, although we cannot rule out the possibility that nuclear DNA may not be the only target. In other words, toxic effects of 1 in tumor cells may be associated with processes at the DNA level.

The finding that the photoactivated 1 is capable of delivering platinum to DNA in the cell nucleus prompted us to examine the binding of 1 to DNA in a cell-free medium. The resulting DNA damage triggers downstream effects including inhibition of replication and transcription, cell cycle arrest, and apoptosis or necrosis (22, 45, 46). The results of these studies were compared with those previously performed with conventional cisplatin and transplatin.

The CT DNA binding experiments carried out in the present work in a cell-free medium indicated that modification reactions resulting in the irreversible coordination of photoactivated 1 were faster than those of cisplatin or transplatin (Table 1). Platinum binding to CT DNA resulting from treatment of DNA with 1 preirradiated for 2 h in the absence of DNA was significantly lower than that resulting from treatment of DNA when 1 was preirradiated for 2 h in the presence of DNA (cf. Figures 2A and 2B).
This observation suggests that free 1 can be transformed into highly reactive species that can be trapped by DNA if it is present in the same solution, whereas photoproducts formed in the absence of DNA become less reactive towards it.

The transcription mapping experiments (Figure 6) indicate that 1 binds to DNA under irradiation conditions at sites similar to those of transplatin, i.e., less regularly than cisplatin and mainly at single guanines and cytosines (i.e., at the preferential DNA binding sites of transplatin and its antitumor analogues (26, 38, 47)). Considerable evidence suggests that the antitumor efficacy of bifunctional platinum compounds is the result of the formation of various types of inter- and intrastrand CLs; however, their relative efficacy remains unknown. The results of this work are consistent with the view that photoactivated 1 forms on DNA ca. only 12% interstrand (intramolecular) CLs (Figure 4, Table 1) and ca. 37% of DNA adducts remain monofunctional even after 24 h (Figure 3, Table 1). The remaining lesions are intrastrand adducts, presumably 1,3-CLs. Thus, it is reasonable to suggest that several aspects of DNA binding mode of photoactivated 1 are similar to those of conventional transplatin (in the dark) (Table 1). On the other hand, it cannot be excluded that identical types of DNA adducts of photoactivated 1 and transplatin can distort DNA conformation differently and can be processed by cellular components differently.

EtBr as a fluorescent probe can be used to characterize DNA binding of small molecules such as platinum antitumor drugs (38, 48). The fluorescence of EtBr is markedly enhanced as a consequence of its intercalation into DNA, but binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by a wide spectrum of DNA-binding platinum drugs. Thus, for instance the modification of DNA by cisplatin or transplatin results in a decrease of EtBr fluorescence intensity as compared with that for non-platinated DNA. The molecules of photoactivated 1 bound to CT DNA sterically block approach of molecules of EtBr to DNA and in this way hinder the intercalation of EtBr to as well, which lowers EtBr fluorescence in comparison with the experiment in which unplatinated DNA was used (Figure 7A). The decrease of EtBr fluorescence in case of DNA modifications by photoactivated 1 was markedly higher than that observed in case of DNA modifications by conventional transplatin or cisplatin in the dark. Comparison
with cisplatin or transplatin suggests that the adducts of 1 photoactivated by UVA or visible light extend over considerably more base pairs around the platination sites than in case of the adducts of transplatin or cisplatin.

The results of experiments in which DNA modifications by photoactivated 1 were probed by EtBr fluorescence (Figure 7A) also suggest that photoactivated 1 forms DNA adducts which cannot be coclustered, from the viewpoint of their capability to inhibit EtBr fluorescence, with those formed by 'conventional' monofunctional platinum(II) complexes, such as cisplatin or transplatin. We also suggest that the pyridine ligands in all, or in a significant fraction of, adducts (mono- and/or bifunctional) might be well positioned to interact with the duplex. The extent of the observed decrease in EtBr fluorescence indicates that the disturbance of the DNA helical structure by photoactivated 1 is not only an effect of strong coordinative platinum binding but has to be explained by an additional interaction of the pyridine ligand(s) of photoactivated 1 with the duplex. The suggestion that the pyridine ligands in the adducts of photoactivated 1 interact with the duplex is further corroborated by the results of DNA unwinding experiments.

Electrophoresis in native agarose gel was used to determine the unwinding induced in negatively supercoiled plasmid by monitoring the degree of supercoiling (Figure 5). The unwinding angle calculated in this way for 1 photoactivated by UVA or visible light was similar, 28 ± 3° or 27 ± 3°, respectively. This unwinding angle is considerably greater than that found for cisplatin or transplatin (13° and 9°, respectively, Table 1). It is reasonable to suggest that the large additional contribution to unwinding is associated with interaction of the pyridine ligand(s) in photoactivated 1 with the duplex. Adducts such as trans-[Pt(py)_2(G)_2] are likely to form. Thus, the large unwinding angle produced by photoactivated 1 is good evidence that the pyridine ligand(s) substantially interacted with duplex DNA upon coordinative binding of platinum. In other words, the unwinding angle observed for photoactivated 1 is consistent with DNA binding that involves a combined mode involving coordination of platinum to a base residue and interaction of pyridine ligand(s) in photoactivated 1 with the duplex. DNA binding that involves a similar combined DNA binding mode as that observed for some cationic platinum(II)
complexes that carry ethidium as a nonleaving group (ethidium is a well known DNA intercalator which unwinds DNA by 26°) or quinoline, such as cis-[Pt(NH$_3$)$_2$Cl(N3/N8-ethidium)]$^+$ or trans-[PtCl$_2$(NH$_3$)(quinoline)] (13, 25). These results obtained with cationic platinum(II) complexes revealed that the intercalating moiety needs to be cis to the Pt-N7 bond in order to interact effectively with the DNA base stack. The analogy between the above-mentioned cationic cis-complex or trans-[PtCl$_2$(NH$_3$)(quinoline)] and photoactivated 1 based on geometric considerations suggests that DNA adducts of the latter may significantly contribute to the unwinding of supercoiled DNA.

The conclusion that the DNA binding mode of photoactivated 1 involves combined coordination of platinum to a base residue and interaction of the pyridine ligand(s) in photoactivated 1 with the duplex may also imply the following. Photoactivated 1 may be capable of forming DNA adducts which induce conformational distortions in DNA which extend over more base pairs than in case of DNA adducts of cisplatin or transplatin. The latter view that adducts of photoactivated 1 distort DNA conformation more than adducts of cisplatin or transplatin is corroborated by the terbium fluorescence data (Figure 7B).

Enhancement of Tb$^{3+}$ ion fluorescence is used to detect base residues in distorted DNA regions (30-32). DNA modification by photoactivated 1 and by cisplatin resulted in an enhancement of the fluorescence, whereas DNA modification by transplatin induced in DNA no conformational distortion detectable by the terbium fluorescence assay (Figure 7B). Thus, the trend in efficiency of DNA adducts of the platinum complexes tested in the present work to enhance Tb$^{3+}$ ion fluorescence was photoactivated 1 $>>$ cisplatin $>$ transplatin. These results along with those of DNA unwinding experiments (Figure 5) support the thesis that DNA adducts of photoactivated 1 induce local conformational alterations in double-stranded DNA that are markedly more extensive than those induced by cisplatin or transplatin.

Studies on the early phases of molecular mechanisms underlying antitumor effects of platinum antitumor drugs have focused on investigations of cellular responses to DNA damage by these metallodrugs (46, 49, 50). Recently attention has been paid to the role of inhibition of transcription elongation by RNA pol II by cisplatin adducts to further understand the mechanism of its biological
effects (20, 51-54). This important component of the mechanism underlying the antitumor effects of platinum drugs has not been hitherto investigated in the case of photoactivatable platinum compounds.

We demonstrate in the present work for the first time (Figure 8) that RNA pol II transcription is highly sensitive to even very low levels of modification on the template DNA by photoactivated 1. Thus, already very low levels of DNA modification by photoactivated 1 may initiate transcription-coupled subpathways leading to apoptosis or necrosis (55).

Importantly, adducts of photoactivated 1 are markedly more effective in inhibiting RNA pol II transcription than those of conventional cisplatin (Figures 8B and 9). This different reduction in transcript production may result from a different level of platination and/or conformational distortions at the promoter, leading to promoter inactivation. The inhibition effects were observed, however, at levels of platination by photoactivated 1 well below those expected to induce such damage \(k_b = 8 \times 10^{-4}\), i.e. only \(~9\) adducts per molecule of template DNA (5764 bp) on average. Hence, the eventuality that the observed different reduction in transcript production may result from a different level of platination and/or conformational distortions at the promoter is unlikely. An alternative possibility is that platinum lesions represent a steric blockage of RNA pol II inhibiting the prolongation of RNA transcription, or that factors essential for RNA pol II initiation may also bind Pt-DNA adducts which do not represent their natural binding sites. Therefore, in the presence of sufficient numbers of Pt-DNA adducts, these factors may become limiting, resulting in an inhibition of transcription initiation due to the factors being sequestered to platinated sites in DNA.

We examined in the present work whether some elements of the transcription complex might be hijacked by DNA adducts of photoactivated 1 more or less than by those of cisplatin using a competition assay (4I) (Figure 8C). The initial addition of control, unplatinated exogenous pUC19 plasmid resulted in an overall increase in the amount of transcript generated by pCMV-Gluc substrate, which increased only slightly upon the further addition of unplatinated exogenous plasmid. Such an increase in transcription efficiency has been already reported (4I) and was attributed to an increase in macromolecular crowding induced by the presence of higher amounts of DNA (4I, 56). We observed in
the present work (Figure 8C), in accord with the previously published results (40, 41), that RNA pol II transcription of the pCMV-Gluc template was markedly reduced by the addition of increasing amounts of cisplatin-modified exogenous plasmid (Figure 8C). Thus, these results are consistent with and support the 'hijacking' hypothesis raised earlier for mononuclear PtII drugs (41, 44, 57, 58) that RNA pol II transcription of template modified by cisplatin is inhibited to a substantial extent due to the transfer of some elements of the transcription complex away from their normal binding sites and in this way interfer with transcription.

A markedly lower inhibition effect was seen if the transcription assay was performed in the presence of exogenous plasmid modified by photoactivated 1 (Figure 8C). Thus, the stronger inhibition of transcription by DNA adducts of photoactivated 1 appears to be due to pronoucnedly more extensive steric blockade of RNA pol II inhibiting the prolongation of RNA transcript and not due to sequestration of elements of the transcription complex to platinated sites in DNA. Thus, this study has revealed that the cause of the stalling of RNA pol II by DNA adducts of photoactivated 1 and cisplatin during transcription is apparently distinctly different providing a basis for a different mechanism of action for photoactivated 1 and cisplatin.

The observation in the present work that transcription elongation by RNA pol II was inhibited by titrating some elements of transcription complex away from their normal binding sites much more by DNA adducts of cisplatin than by those of photoactivated 1 (Figure 8C) deserves further discussion. Many transcription factors are HMG-domain proteins (HMG = high mobility group). These proteins have been shown to recognize and bind with a strong affinity to structural motifs in DNA that involve directional rigid bends of the longitudinal axis of this double-helical nucleic acid. Major DNA adducts of cisplatin (intrastrand CLs between neighboring purine residues which represent ~90% of all DNA adducts of cisplatin (16)) produce in DNA a stable directional curvature (~40° toward major groove of DNA) (44, 59). Therefore, this bending apparently represents an important structural motif responsible for a high affinity of many transcription factors to DNA modified by cisplatin. In contrast, DNA adducts of photoactivated 1 (presumably similar to those formed by conventional transplatin, i.e. mainly 1,3-
intrastrand CLs, monofunctional adducts and to a much lesser extent interstrand CLs, Table 1) rather produce in DNA flexible and relatively small nondirectional bends so that these adducts are not recognized by HMG-domain proteins (60-62). Hence, DNA adducts of photoactivated 1 may lack, in contrast to DNA adducts of cisplatin, a high-affinity structural motif which would attract transcription factors.

In conclusion, the Pt\textsuperscript{IV} diazido complex \textit{trans,trans,trans-}[Pt(N\textsubscript{3})\textsubscript{2}(OH)\textsubscript{2}(pyridine)\textsubscript{2}] (1) is unreactive in the dark but a potent anticancer drug when photoactivated, not only by UVA but also by visible light. Complex 1 when photoactivated accumulates in tumor cells, penetrates the nucleus and binds strongly to nuclear DNA under conditions in which it exhibits cytotoxicity. The nature of the DNA adducts, including conformational alterations, induced by photoactivated 1 are distinctly different from those produced in DNA by conventional cisplatin or transplatin. In addition, another critical difference between photoactivated 1 and cisplatin emerged with respect to the ability of their DNA adducts to inhibit transcription of DNA by stalling RNA pol II. Photoactivated 1 proves to be a significantly more potent inhibitor of RNA synthesis than cisplatin (Figures 8 and 9, Table 1). Inhibition of DNA transcription is considered to be a major mediator of the cell kill effect of cisplatin (20, 51, 63). The observation that major adducts of photoactivated 1 are able to efficiently stall RNA pol II suggests that transcription inhibition may contribute to the cytotoxicity levels observed for photoactivated 1 (3). Hence, DNA adducts of 1 could trigger a number of downstream cellular effects different from those triggered in cancer cells by DNA adducts of cisplatin. This might lead to the therapeutic effects that could radically improve chemotherapy by platinum complexes. These findings do not rule out the possibility that other photodecomposition products also contribute to the cytotoxicity of complex 1. These might include for example the release of azide, reactions of azido radicals or nitrenes with biomolecules. Such possibilities together with the present findings may explain the different pharmacological effects of photoactivated 1 and conventional cisplatin and thereby provide new insights into mechanisms associated with the antitumor effects of platinum complexes photoactivated by UVA and/or visible light (3).
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ABBREVIATIONS

bp, base pair; cisplatin, cis-Pt(NH₃)₂Cl₂; CL, cross-link; CT, calf-thymus; DTT, dithiothreitol; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectroscopy; PAA, polyacrylamide; PDT, photodynamic therapy; [PtCl(dien)]Cl, chloridodiethylenetriamineplatinum(II) chloride; r₀, the number of molecules of the platinum complex coordinatively bound per nucleotide residue; r₁, the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA; t₅₀%, the time at which the binding reached 50%; transplatin, trans-Pt(NH₃)₂Cl₂; TU, thiourea.
REFERENCES


Table 1. Summary of DNA Binding Characteristics of Cisplatin, Transplatin and Photoactivated 1

<table>
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<tr>
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<sup>a</sup>This work.  <sup>b</sup>The time at which the binding reached 50%.  <sup>c</sup>Bancroft et al. (35).  <sup>d</sup>Fichtinger-Schepman et al. (16).  <sup>e</sup>Eastman (64).  <sup>f</sup>Brabec and Leng (26).  <sup>g</sup>Keck and Lippard (25).  <sup>h</sup>Not determined.
FIGURE CAPTIONS

Figure 1. Structure of \(\text{trans},\text{trans},\text{trans} - \{\text{Pt(N}_3)_2(\text{OH})_2(\text{pyridine})_2\}\) (1) used in this work.

Figure 2. Kinetics of the reaction of 1 photoactivated by UVA (A,B) or visible (C) light with double-helical CT DNA. A. The samples were irradiated \(\left(\lambda_{\text{max}} = 365 \text{ nm}\right)\) continuously starting immediately after mixing DNA with the platinum complex (open squares), incubated in the dark (full squares), or solutions of the platinum complex were first preirradiated for 2 h in the absence of DNA and then added to DNA (open circles). B. The samples were irradiated continuously starting immediately after mixing DNA with the platinum complex for 30 min (triangles), 1 h (inverted triangles), 2 h (circles) and 7 h (squares) and then incubation was continued in the dark up to 24 h. C. The samples were irradiated \(\left(\lambda_{\text{max}} = 458 \text{ nm}\right)\) continuously starting immediately after mixing DNA with the platinum complex. Solutions of DNA at a concentration of \(32 \mu g \text{ mL}^{-1}\) were incubated with the platinum complex at the concentration of \(5 \mu M\) (the value of \(r_i\) was 0.05) in NaClO\(_4\) (10 mM) at 37 °C. Points represent mean value of three independent experiments ± SD.

Figure 3. Kinetics of reaction of 1 photoactivated by (A) UVA, or (B) visible light with double-helical CT DNA under irradiation conditions (irradiation for 1 h and subsequent incubation for 23 h in the dark) at \(r_i = 0.05\) in NaClO\(_4\) (10 mM) at 37 °C. DNA concentration was \(80 \mu g \text{ mL}^{-1}\). Reactions were stopped with thiourea (10 mM, 10 min) (full squares) or NaCl (0.1 M, -20 °C) (open squares), and the amount of platinum associated with DNA was determined by FAAS. For other details, see the text.

Figure 4. Formation of interstrand (intramolecular) cross-links by 1 photoactivated by UVA or visible light in pSP73 plasmid linearized by EcoRI. Solutions of linearized plasmid (50 \(\mu g \text{ mL}^{-1}\)) were incubated with the platinum complex under irradiation conditions (for 1 h and subsequently incubated in
the dark for additional 23 h) at various r\textsubscript{i} values in NaClO\textsubscript{4} (10 mM) at 37 °C. Autoradiogram of denaturing 1% agarose gel of linearized DNA which was 3'-end labeled and nonmodified (lanes 1, 2, and 12) or modified by 1 photoactivated by UVA (lanes 4-11) or visible (lanes 13-15) light. Interstrand cross-linked DNA appears as the top bands (marked as ICL) migrating on the gel more slowly than single-stranded DNA (contained in the bottom bands and marked as ss). The fragment was nonplatinated and nonirradiated (kept in the dark) (lane 1), nonplatinated and UVA-irradiated (lane 2), modified by 1 photoactivated by UVA radiation so that the resulting r\textsubscript{b} values were 0.0003, 0.0004, 0.0005, 0.0006, 0.0007, 0.0008, 0.0009, and 0.001 (lanes 4-11, respectively), nonplatinated and irradiated by visible light (lane 12) or modified by 1 photoactivated by visible light so that the resulting r\textsubscript{b} values were 0.0003, 0.0006, and 0.0009 (lanes 13-15, respectively). DNA fragment modified by cisplatin in the dark to r\textsubscript{b} = 0.001 is included as well (lane 3).

**Figure 5.** Unwinding of supercoiled pUC19 plasmid DNA by 1 photoactivated by UVA. The top bands correspond to the nicked form of plasmid and the bottom bands to the closed, negatively-supercoiled plasmid. The plasmid was incubated with 1 under irradiation conditions (for 1 h and subsequently incubated in the dark for additional 23 h) at various r\textsubscript{i} values in NaClO\textsubscript{4} (10 mM) at 37 °C. Lane C, control, nonplatinated and nonirradiated DNA; lane C\textsubscript{UVA}, control, nonplatinated DNA (r\textsubscript{b} = 0); lanes 1-8, DNA modified by photoactivated 1 so that the resulting r\textsubscript{b} values were 0.005, 0.01, 0.015, 0.02, 0.025, 0.003, 0.035, and 0.04, respectively.

**Figure 6.** RNA synthesis by T7 RNA polymerase on the NdeI/HpaI fragment of pSP73KB plasmid modified by cisplatin, transplatin (in the dark) or by 1 photoactivated by UVA or visible light (the samples were irradiated for 1 h and subsequently incubated in the dark for additional 23 h). A. Autoradiogram of 6% polyacrylamide/8M urea sequencing gel. Lanes: C(dark), control, nonplatinated
template (incubated in the dark for 24 h); C(UVA), control, nonplatinated template irradiated by UVA (in the absence of platinum complex); C(VIS), control, nonplatinated template irradiated by visible light (in the absence of platinum complex); cisPt, transPt, template modified by cisplatin or transplatin, respectively at \( r_b = 0.01 \) in the dark; I(UVA), I(VIS), template treated with I photoactivated by UVA or visible light, respectively so that the level of the modification corresponded to \( r_b = 0.01 \); C, G, U, and A, chain terminated marker RNAs. B. Sequence of NdeI/HpaI fragment of pSP73KB plasmid. Arrow indicates start of T7 RNA polymerase, which was used as a template. Numbers correspond to nucleotide numbering in sequence of pSP73KB plasmid.

**Figure 7.** Fluorescence measurements. A. Dependencies of ethidium bromide fluorescence on \( r_b \) for double-helical CT DNA modified by various platinum complexes in NaClO\(_4\) (10 mM) at 37°C for 48 h. (■) Cisplatin; (●) transplatin; (□), I photoactivated by UVA radiation. B. Change in the relative fluorescence of Tb\(^{3+}\) ion produced by its binding to double-helical CT DNA modified by cisplatin (■), transplatin (●), or I photoactivated by UVA radiation (□) at various \( r_b \). Fluorescence of untreated DNA was arbitrarily set at unity. CT DNA was incubated with I under irradiation conditions (for 1 h and subsequently incubated in the dark for additional 23 h) at various \( r_i \) values in NaClO\(_4\) (10 mM) at 37 °C. It was verified that irradiation of CT DNA by UVA or visible light in the absence of I had no effect on EtBr or terbium fluorescence.

**Figure 8.** Inhibition of RNA polymerase II transcription by DNA adducts of cisplatin (formed in the dark) or by I photoactivated by UVA or visible light (the samples were irradiated for 1 h and subsequently incubated in the dark for additional 23 h). A) Autoradiogram of the 8% PAA/8 M urea denaturing gel. Lanes: 1, 5, and 9, control, unplatinated pCMV-Gluc substrate that was kept in the dark or irradiated by UVA or visible light, respectively; 2, 3, and 4, pCMV-Gluc substrate modified with...
cisplatin at \( r_b = 8 \times 10^{-4} \), \( 1.6 \times 10^{-3} \) and \( 3.2 \times 10^{-3} \), respectively; 6,7, and 8, pCMV-Gluc substrate modified 1 photoactivated by UVA at \( r_b = 6.6 \times 10^{-4} \), \( 1.32 \times 10^{-3} \) and \( 3.3 \times 10^{-3} \), respectively; 10, 11 and 12, pCMV-Gluc substrate modified 1 photoactivated by visible light at \( r_b = 5.2 \times 10^{-4} \), \( 1.1 \times 10^{-3} \) and \( 3.0 \times 10^{-3} \), respectively. B) Quantitative assessment. The relative transcription was assessed as follows: The amount of full length transcript at each \( r_b \) was quantified (in % of total radioactivity in the lane) and calculated as the percentage of that generated by the control, undamaged template. Data represent results of two independent experiments and are expressed as mean percentages ±SEM. (□) cisplatin; (■) 1 photoactivated by UVA radiation; (▼) 1 photoactivated by visible light. C. Inhibition of RNA polymerase II transcription of pCMV-Gluc substrate by the addition of increasing amount of exogenously platinated pUC19 DNA. The amount of full-length transcript in each lane is expressed as a mean fraction (±SEM) of that generated in the absence of exogenously added DNA (white bar). Black bars: transcription in the presence of undamaged DNA. Dark gray bars: transcription in the presence of cisplatin modified DNA. Light gray bars: transcription in the presence of DNA modified with 1 photoactivated by UVA radiation.

Figure 9. A. Scheme illustrating the preparation of DNA templates containing single, site-specific adducts of photoactivated 1 (1,3-GTG intrastrand cross-link) (top) or cisplatin (1,2-GG intrastrand cross-link) (bottom) indicating the locations of T7 and SP6 promoters and platinum adduct. The arrows indicate the sites where the top strands of the substrates were ligated together from three 23-nt oligonucleotides, central oligonucleotide was platinated. B. Analysis of termination of RNA synthesis by T7 (left panel) or SP6 (right panel) RNA polymerases by DNA adducts of cisplatin or photoactivated 1. Lanes: no Pt, control, nonplatinated template; Pt, substrate containing site specific intrastrand cross-link of 1 or cisplatin in GTG or GG sequence, respectively; dC, chain terminated marker showing position of guanines on the template, 3'-deoxyribocytidine was added to the reaction. For other details, see Experimental Procedures.
Figure 1

Figure 2
Figure 3

A

% Pt bound

Time (h)

B

% Pt bound

Time (h)

Figure 4

ICLs

ss

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Figure 5

Figure 6

A

B
Figure 7

(A) Relative fluorescence vs. $r_b$
(B) Relative fluorescence vs. $r_b$

Figure 8

(A) Western blot images

(B) Full length transcript (%) vs. $r_b$

(C) Relative transcription vs. DNA added (ng)
Figure 9

(A) 5'-ATTTAGGTTGACCTATAGAACACCTCCTCTCCTGAGTACCCATAGATGAGTTAATTA -3'
3'-TAATTCACGTGATATCTTTGGAGAGGAGACAGAGAGAGAGAGGAGATATCTCAGCTAAAT -5'

SP6 RNA polymerase

T7 RNA polymerase

(B) T7

GG  dC  Pt  noPt  GTG  dC  Pt  noPt

SP6

GG  Pt  noPt  GTG  Pt  noPt