Transplatin Is Cytotoxic When Photoactivated: Enhanced Formation of DNA Cross-Links

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It is well-known that although cisplatin, [*cis*-[$PtCl_2(NH_3)_2$], is an anticancer drug, its isomer transplatin is not cytotoxic. Here we show that transplatin is almost as cytotoxic as cisplatin when treated cells (human keratinocytes HaCaT and ovarian cancer A2780 cells) are irradiated with UVA light (50 min, 1.77 mW cm^{-2}). Chemical studies show that light activates both chloride ligands of transplatin, and experiments on pSP73 plasmid DNA and a 23 base-pair DNA duplex show that irradiation can greatly enhance formation of interstrand cross-links and of DNA−protein cross-links (which are not formed in the dark). Comet assays showed that UVA irradiation of transplatin-treated cells resulted in an increased inhibition of H_2O_2-induced DNA migration, supporting the conclusion that the cytotoxicity of photoactivated transplatin is mainly due to formation of DNA interstrand and DNA−protein cross-links.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)[a]] is currently one of the leading drugs used against cancer. Three other structurally related platinum drugs (carboplatin [*cis*-diamminecyclobutanedicarboxylatoplatinum(II)], nedaplatin [*cis*-diammineglycolatoplatinum(II)], and oxaliplatin [(1*R*,2*R*-diamminocyclohexane)-oxalatoplatinum(II)]) have also entered widespread clinical use,[1,2] and the importance of this class of drugs is emphasized by the fact that there is hardly any clinical regimen of combination chemotherapy today that does not contain cisplatin or another platinum drug. On the other hand, despite its success, cisplatin has several disadvantages, such as inherent and acquired resistance, and side effects.[3] The drawbacks coupled with toxicity of cisplatin and its analogues have been the impetus for the development of platinum drugs with improved pharmacological properties and a broader range of antitumor activity.

The design of photoactivatable metallodrugs is a new approach in chemotherapy of cancer that is now extensively studied. One of the strategies in this field is based on administration of prodrugs, which can be selectively activated in tumor tissue, thereby minimizing the side effects of chemotherapy. Photoactive platinum(IV) analogues of antitumor cisplatin or its clinically ineffective trans isomer (transplatin) were recently synthesized and were found to be as cytotoxic as cisplatin upon irradiation with UVA light.[4,5] Therefore, it is of special interest to examine whether parent cisplatin and transplatin are also affected by irradiation with light to the extent that their biological, including cytotoxic, properties are altered. We have found in the present work that the toxicity of cisplatin in tumor cells is affected by irradiation only very slightly whereas the cytoxicity of transplatin is markedly enhanced. In order to at least identify some factors responsible for the transformation by light of an inefficient platinum complex into a cytotoxic drug, we have also examined modifications of DNA by transplatin in both cell-free media and cells under irradiation conditions by means of the methods of molecular biology and biophysics. These studies were undertaken since DNA is the major target of the cytotoxic effects of platinum complexes.[6,7]

Results and Discussion

Phototoxicity in Cell Culture. HaCaT keratinocytes and A2780 and A2780CIS ovarian carcinoma cells were exposed to transplatin or cisplatin for 1 h followed by a 50 min irradiation or sham irradiation. Cell viability was determined by the uptake of neutral red dye. An IC_{50} value could not be determined for sham-irradiated transplatin in HaCaT cells, as cell viability was 77.6% ± 4.4 at the maximal dose of transplatin (333 μM) used. The combination of UVA and transplatin was more toxic to HaCaT cells than transplatin alone (Figure 1A, Table 1). Cisplatin in the dark was more cytotoxic than its trans analogue, but in contrast, irradiation of cisplatin did not increase its toxicity (Figure 1B, Table 1). The dose of UVA used in the experiments was low; 5 J cm^{-2} of UVA corresponds to approximately 30−60 min of midday sun (Scotland). The sensitivity of HaCaT keratinocytes to this dose of UV radiation is similar to that of primary keratinocytes: cell viability (+UVA/−UVA) at 5 J cm^{-2} was 86.0% ± 3.6%. An irradiation-dependent dose effect was also observed; when the UVA dose was increased to 10 J cm^{-2}, the IC_{50} value decreased to 115.3 μM in HaCaT cells. No IC_{50} value could be obtained for identically controlled cells within the dose range (Table 1).

A2780 and A2780CIS ovarian carcinoma cells are more sensitive to UVA light than HaCaT keratinocytes; however, a similar trend was seen in these cells. Irradiation of cisplatin had no effect on the toxicity of this drug in both cell lines, but irradiation of transplatin increased the effectiveness of transplatin in A2780 cells by 2-fold.

DNA Binding in Cell-Free Media. We examined first DNA binding of transplatin in cell-free media when the reaction mixture of DNA with transplatin was irradiated. Solutions of calf thymus (CT) DNA at a concentration of 0.32 mg/mL were

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[a] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); CL, cross-link; CPZ, chlorpromazine; CT, calf thymus; DPCL, DNA−protein cross-link; FAAS, flameless atomic absorption spectrophotometry; PAGE, polyacrylamide gel electrophoresis; r_b, the number of molecules of the platinum compound bound per nucleotide residue; r_i, the molar ratio of free platinum complex to nucleotide-phosphates at the onset of incubation with DNA; SDS, sodium dodecyl sulfate; transplatin, *trans*-diamminedichloroplatinum(II); TU, thiourea.

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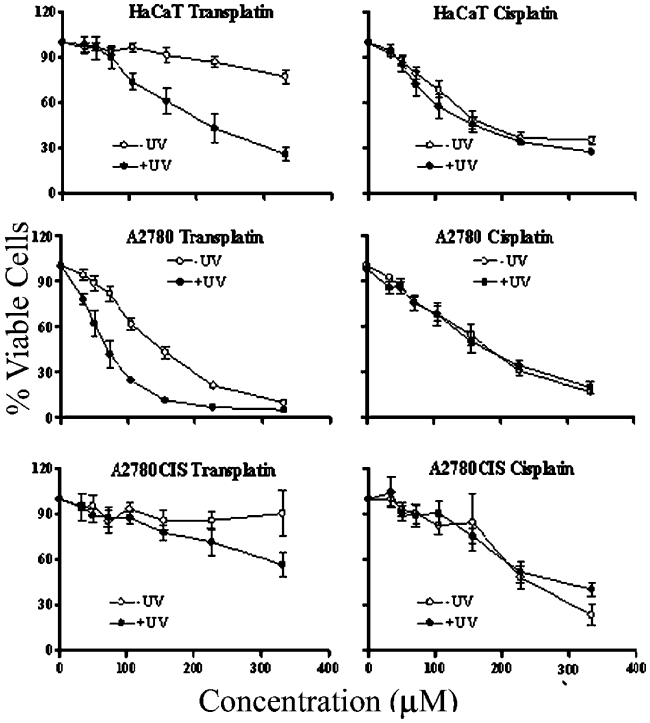

Figure 1. Phototoxicity of transplatin and cisplatin in HaCaT keratinocytes and A2780 and A2780CIS ovarian carcinoma cells. Cells were incubated with test compound for 1 h prior to irradiation with 5 J cm^{-2} UVA, or sham irradiated. After this time, test compounds were removed and the cells allowed to recover in complete medium for 24 h before assaying with neutral red. The experiments were performed in triplicate and repeated three times. Data points represent the mean ± SE of each test condition.

Table 1. Toxicity of Transplatin and Cisplatin[a]

test compound	IC_{50} value (μM) +UV	IC_{50} value (μM) −UV
	HaCaT Cells	
transplatin (5 J cm^{-2})	195	>333[b]
transplatin (10 J cm^{-2})	115.1	>333[b]
cisplatin (5 J cm^{-2})	144	173
	A2780 Cells	
transplatin (5 J cm^{-2})	60	131
cisplatin (5 J cm^{-2})	151	152
	A2780CIS Cells	
transplatin (5 J cm^{-2})	>333[b]	>333[b]
cisplatin (5 J cm^{-2})	261	229

[a] For other details, see the legend to Figure 1; the mean IC_{50} value of photoactivated CPZ was 9 μM for HaCaT cells, 5.7 μM for A2780 cells, and 4.9 μM for A2780CIS cells. [b] The maximum added dose was 333.3 μM.

incubated with transplatin at r_i values in the range of 0.01−0.1 in 10 mM $NaClO_4$ at 37 °C (r_i is defined as the molar ratio of free platinum complex to nucleotide-phosphates at the onset of incubation with DNA). The experiments were performed under conditions when the reaction mixture of DNA with transplatin was UVA irradiated for various time intervals as described in the experimental part. Times of the incubation of DNA with transplatin longer than 5 h have not been used since DNA was degraded at the longer times of irradiation. At various time intervals an aliquot of the reaction mixture was withdrawn and assayed by flameless atomic absorption spectrophotometry (FAAS) for platinum bound to DNA (r_b, which is defined as the number of molecules of the platinum compound bound per nucleotide residue). The amount of platinum coordinated to DNA increased with time with a rate identical to that found for the reaction of DNA with transplatin in the dark; after 5 h approximately 70% transplatin was quantitatively bound. Importantly, the analytical method used monitors the covalent attachment of platinum complexes to DNA (so that the results are not affected by the subsequent closure of monofunctional adducts to bifunctional lesions).

The next studies were, therefore, aimed at quantification of monofunctional platinum adducts by thiourea (TU) assay.[8,9] Cisplatin, transplatin, and analogous bifunctional platinum compounds coordinate to DNA in a two-step process, forming first the monofunctional adducts preferentially at guanine residues, which subsequently close to bifunctional lesions.[10,11] TU is used to labilize monofunctionally bound transplatin from DNA. The displacement of transplatin is initiated by coordination of TU trans to the nucleobase. Because of the strong *trans* effect of sulfur in TU, the nucleobase nitrogen−platinum bond is weakened and thus becomes susceptible to further substitution reactions. Consequently, transplatin in monofunctional DNA adducts is effectively removed, whereas bifunctional adducts of transplatin are resistant to TU treatment.[8]

Double-stranded CT DNA at the concentration of 0.022 mg/mL was incubated with transplatin in the dark or under irradiation conditions at a drug-to-nucleotide ratio of $r_i = 0.08$ in 10 mM $NaClO_4$ at 37 °C. After 5 h, the reaction was stopped by adjusting the NaCl concentration to 0.2 M and by immediate cooling to −20 °C. In parallel experiments, the reaction was stopped by addition of 10 mM TU solutions. These samples were incubated for 10 min at 37 °C and then quickly cooled to −20 °C. The samples were then exhaustively dialyzed against 0.2 M NaCl and subsequently against H_2O at 4 °C, and the platinum content was determined by FAAS. TU displaced ca. 85% of the transplatin species from DNA modified by this platinum compound in the dark within 5 h in accord with previously published data.[8,9] Interestingly, TU displaced ca. 75% transplatin from DNA modified by this platinum compound under irradiation conditions. It implies that the amount of bifunctional adducts formed under irradiation conditions was somewhat higher than that found for the mixture of DNA and transplatin kept in the dark. It was verified that 5−60 min incubations with 10 mM TU gave the same results as those described above.

Bifunctional DNA adducts of transplatin are intrastrand and interstrand cross-links (CLs), and theoretically the irradiation may affect proportional representation of these two types of CLs. The amounts of interstrand CLs formed by transplatin were measured in linearized plasmid DNA incubated with transplatin for various times. The bands corresponding to more slowly migrating interstrand-cross-linked fragments were already noticed if transplatin was incubated with linearized DNA in the dark for 1 h (Figure 2A, lane 12). The intensity of the more slowly migrating band (corresponding to interstrand-cross-linked DNA) increased with the growing time of the modification. After 5 h, the percentage of DNA interstrands cross-linked by transplatin in the dark reached 11% (Figure 2B). We have determined in parallel the amount of platinum bound to DNA (r_b) at each time interval by FAAS. The frequency of interstrand CLs produced by transplatin (the amount of interstrand CLs per one molecule of this platinum complex bound to DNA multiplied by 100) in the dark also increased with time (Table 2) and reached 3.5% after 5 h, in accordance with previously published data.[12]

The same experiments were performed under identical conditions when the reaction mixture of DNA with transplatin was UVA-irradiated for various time intervals as described in

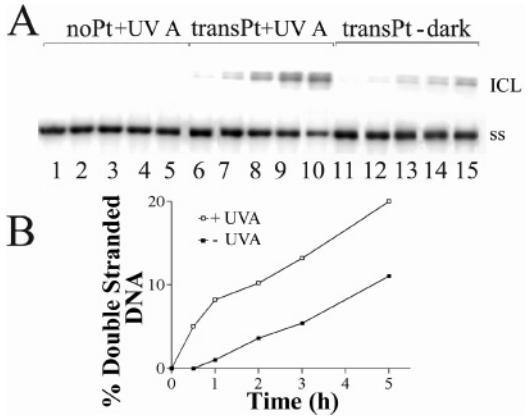

Figure 2. The formation of DNA interstrand CLs by transplatin in pSP73 plasmid linearized by *Eco*RI. (A) Autoradiogram of denaturing 1% agarose gel of linearized DNA which was 3′-end labeled, left unplatinated or mixed with transplatin at $r_i = 5 \times 10^{-4}$ and incubated at various time intervals at 37 °C. The interstrand cross-linked DNA appears as the top bands (marked as ICL) migrating in the gel more slowly than the single-stranded DNA (contained in the bottom bands and marked as ss). The fragment was nonplatinated (control) (lanes 1−5) or modified by transplatin under irradiation conditions (lanes 6−10) or in the dark (lanes 11−15). DNA was mixed with transplatin and irradiated with 4.3 mW cm^{-2} UVA or sham irradiated for: 0.5 h, lanes 1, 6, 11; 1 h, lanes 2, 7, 12; 2 h, lanes 3, 8, 13; 3 h, lanes 4, 9, 14; and 5 h, lanes 5, 10, 15. (B) Kinetics of the cross-linking by irradiated transplatin (■) or sham irradiated (▲) in the linearized DNA at $r_i = 5 \times 10^{-4}$. The cross-linking reaction was stopped at the time intervals indicated on the *x*-axis by adjusting the NaOH concentration to 30 mM and cooling the samples to −20 °C.

Table 2. Binding of Transplatin to pSP73 Plasmid Linearized by *EcoRI*[a] and Frequency of Interstrand Cross-Linking (% ICL/adduct)[b]

time[c] (hours)	r_b 10^4 [d]	% ICL/adduct in the dark	% ICL/adduct under irradiation conditions
0.5	2.5	0	4.2
1	3.3	0.6	5.2
2	4.1	2.3	5.3
3	4.4	2.5	6.3
5	4.5	3.5	11.5

[a] Binding reaction proceeded in 10 mM $NaClO_4$ at 37 °C; r_i was 5×10^{-4}. [b] Frequency of interstrand cross-linking is defined as the amount of interstrand CLs per one molecule of transplatin bound to DNA multiplied by 100. [c] Time of the incubation of DNA with transplatin. [d] Amount of platinum bound to DNA was identical in the dark and under irradiation conditions.

the Experimental Section. The times longer than 5 h for the incubation of DNA and transplatin have not been used since DNA was degraded at longer times of irradiation. The percentage of DNA interstrands cross-linked by transplatin under irradiation conditions also increased with time but more rapidly compared to the cross-linking in the dark and reached 20% after 5 h (Figure 2B), which corresponded to a frequency of interstrand CLs of 11%. Thus, the efficiency of irradiated transplatin to form interstrand CLs in DNA is enhanced ca. 3 times, whereas its efficiency to form intrastrand CLs is enhanced only slightly (1.2 times).

In summary, the observation that monofunctional adducts of transplatin evolved to bifunctional lesions at a higher rate if the reaction mixture of transplatin and DNA was irradiated was apparently due to the ability of irradiated transplatin to form mainly interstrand CLs more readily (Table 3).

Transplatin was also investigated for its ability to form ternary DNA−Pt−protein complexes (DNA−protein CLs, DPCLs). The 23-bp duplex was mixed with histone H1 or NF-κB (p50 dimer)

Table 3. Binding of Transplatin to DNA and Frequency of the Adducts[a]

	in the dark	UVA irradiated
monofunctional adducts (%)[b]	85	75
interstrand CLs (%)[c]	3.5	11
intrastrand CLs (%)[d]	11.5	14

[a] Frequency of the adduct is defined as the amount of the adduct per one molecule of transplatin bound to DNA. [b] Binding to CT DNA proceeded in 10 mM $NaClO_4$ at 37 °C for 5 h; r_i was 0.08. [c] Binding to pSP73 plasmid linearized by *Eco*RI proceeded in 10 mM $NaClO_4$ at 37 °C for 5 h; r_i was 5×10^{-4}. [d] Frequency of intrastrand CLs was determined as: frequency of bifunctional adducts minus frequency of interstrand CLs.

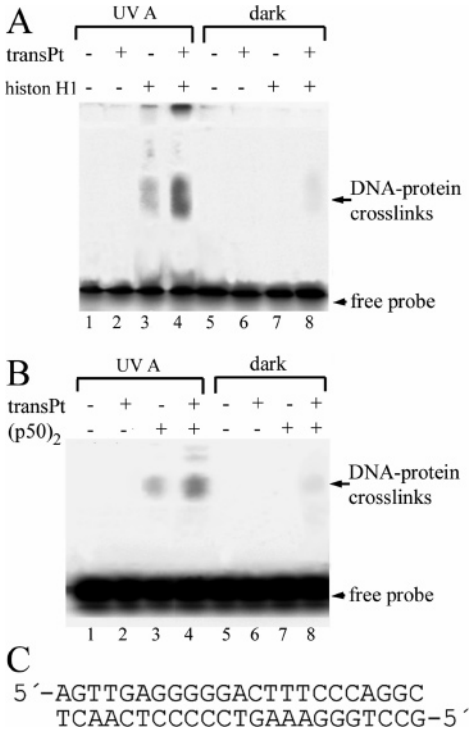

Figure 3. DNA−protein cross-linking by transplatin. Cross-linking DNA and histone H1 (A) or NF-κB (B). The samples containing DNA, transplatin, and the protein were irradiated with UVA (lanes 1−4) or sham irradiated (lanes 5−8) for 5 h. The 23-bp duplex (the central part of its nucleotide sequence shown in Figure 3C) was mixed with transplatin (lanes 2, 4, 6, 8) (the molar ratio platinum/duplex was 1), histone H1 (Figure 3A, lanes 3, 4, 7, 8) or NF-κB (Figure 3B, lanes 3, 4, 7, 8) (the molar ratio protein/duplex was 5). Ternary DNA−Pt−protein cross-linking efficiency was assessed by SDS/PAGE shift assay. (C) The nucleotide sequence of the duplex used in this study.

proteins (the molar ratio protein/duplex was 5) and simultaneously also with transplatin (the molar ratio platinum/duplex was 1). The mixture was incubated for 5 h in the dark. Ternary DNA−Pt−protein cross-linking efficiency was assessed by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) shift assay. No fractions with significantly retarded mobility, which would indicate the formation of the ternary DNA−Pt−protein complexes, by SDS/PAGE were detected (Figures 3A,B, lanes 8). On the other hand, if the same experiments were performed under identical conditions, but the reaction mixture of DNA, transplatin, and protein (histone H1 or NF-κB) was irradiated for 5 h as described in the Experimental Section, fractions were detected with significantly retarded mobility by SDS/PAGE (Figures 3A,B, lanes 4) compared with that of the free probe unplatinated or modified by transplatin (Figures 3A,B, lanes 1, 2, respectively). This fraction was eliminated after treatment with NaCN or proteinase K converting it to that of the unmodified probe (not shown).

These results suggest that the species is a DPCL tethered by platinum−DNA and platinum−protein covalent bonds. While the proteinase K and NaCN experiments clearly indicate that protein is the species cross-linked to DNA, the amino acids participating in the cross-linking reaction have not been determined. It should be pointed out that the DPCLs were also formed if the mixture of the protein and DNA was irradiated under the same conditions as in previous experiments in the absence of transplatin, but the amount of these CLs was markedly lower compared to the cross-linking in the presence of transplatin (ca. 10 times, Figures 3A,B, lanes 3). Thus, while transplatin forms no DPCLs in the dark, these ternary complexes are formed if the reaction mixture containing DNA, transplatin, and protein is irradiated.

DNA Cross-Linking in Cells. We have also examined whether the irradiation results in the enhancement of interstrand CLs and DPCLs in cells, i.e., when cells are treated with transplatin under irradiation conditions. We employed for this purpose the single cell gel electrophoresis assay (comet assay). This assay is a rapid and sensitive method of studying DNA damage and repair in individual cells.[13,14] Although mainly used to identify alkali-sensitive damage such as strand breaks, the method has recently been adapted to study cross-linking agents, including cisplatin.[15−20] To achieve this, after the cells have been incubated with the cross-linking agent they are treated with a clastogen (γ-rays, X-rays, or H_2O_2), which introduces a number of strand breaks into the DNA. Interstrand CLs can interfere with the alkaline denaturation of the DNA, thus antagonizing the effects of the clastogen, by making the DNA molecule too big to migrate during electrophoresis. The comet assay has been used to identify compounds that produce mainly interstrand CLs and also DPCLs[21] but cannot discriminate between these adducts.

In the dark, neither cisplatin nor transplatin increased detectable alkali-sensitive damage (data not shown). Exposure to 50 μM H_2O_2 (5 min, 4 °C) produced significant DNA migration (78.5 ± 5.1% tail DNA) compared to background (2.5 ± 0.4% tail DNA). When cells were treated with Pt compounds before H_2O_2 treatment, migration was significantly inhibited (Figure 4A), suggesting the formation of CLs. Transplatin was not as effective as cisplatin in preventing H_2O_2-induced DNA migration under these conditions, and there was good correlation between cytotoxicity in the dark and the extent to which DNA migration was antagonized (cisplatin: $r^2 = 0.91$; transplatin $r^2 = 0.97$). Irradiation of transplatin-treated cells increased the extent to which H_2O_2-induced DNA migration was antagonized, mirroring the results obtained with the photocytotoxicity assay (Figure 4B; $r^2 = 0.92$). The effects of irradiation on cisplatin were not examined.

NMR Spectroscopic Studies. The results of the present work indicate that transplatin forms considerably more CLs in DNA under irradiation conditions than in the dark (*vide supra*). Therefore, we also investigated by NMR spectroscopy the propensity of transplatin to lose both chloride ligands upon irradiation, which is a prerequisite of the formation of bifunctional DNA adducts in DNA by this platinum complex.[22] Two identical samples containing transplatin (1 mM) and 5′-GMP (1 mM) were prepared in 90% H_2O/10% D_2O. One sample was irradiated with UVA light while the other was kept in the dark. The 1D ^{1}H and 2D [^{1}H,^{15}N] HSQC NMR spectra were recorded soon after preparation and then after 1 and 3 h. The spectra of the two samples were very different after 3 h (Figure 5). The 2D spectrum of the irradiated sample contained many more

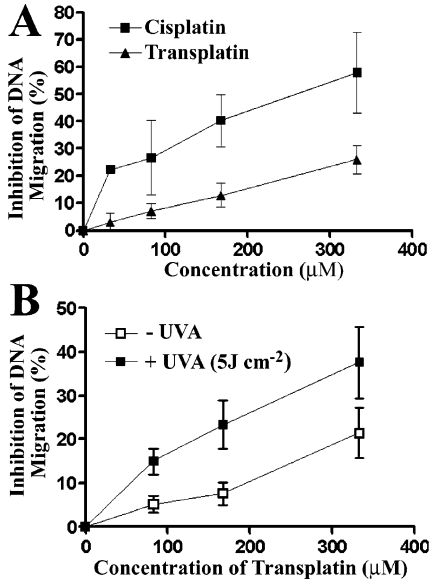

Figure 4. Cross-linking in HaCaT cells measured by the comet assay. Cells were treated as for Figure 1. Results were generated as described in the Experimental Section. The experiments were performed in duplicate and repeated three times. Data points represent the mean ± SE of each test condition.

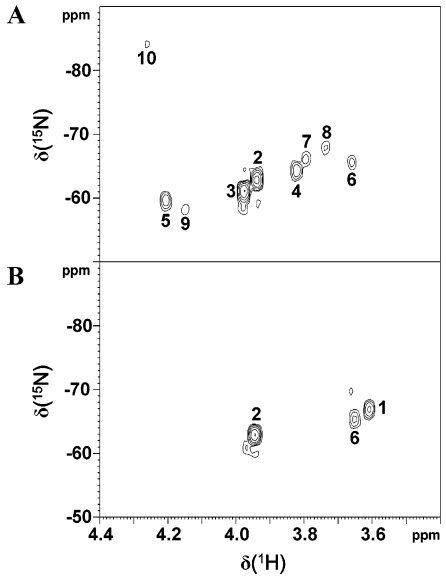

Figure 5. 2D [^{1}H,^{15}N] HSQC NMR spectra of transplatin (1 mM) and 5′-GMP (1 mM) after 3 h UVA irradiation (1.5 mW cm^{-2}) (A) and 3 h in the dark (B). Peak assignments: (**1**) ^{15}N-transplatin, (**2**) *trans*-[PtCl($^{15}NH_3$)$_2$(5′-GMP)], (**3**) *trans*-[Pt($^{15}NH_3$)$_2$(OH$_2$)(5′-GMP)], and (**4**) *trans*-[Pt($^{15}NH_3$)$_2$(5′-GMP)$_2$]. Peaks **4**, **6**−**9** are unassigned but have ^{15}N chemical shifts consistent with H_3N−Pt *trans* to N or Cl, whereas peak **10** appears to be assignable to a H_3N−Pt−O species.

cross-peaks than that of the sample kept in the dark, indicating that transplatin is photoactive upon UVA irradiation.

One of the major new peaks in the irradiated sample is assignable to *trans*-[Pt($^{15}NH_3$)$_2$(5′-GMP)(OH$_2$)]$^{2+}$ (**3**);[23] the other major peak (**2**) is the monochloro species *trans*-[Pt($^{15}NH_3$)$_2$(5′-GMP)Cl]$^+$ which is the main species present in the dark sample after 3 h (Table 4). The irradiated sample also contains a peak for the bis-GMP adduct *trans*-[Pt($^{15}NH_3$)$_2$(5′-GMP)$_2$]$^{2+}$ (**5**).[5,23] No peak for transplatin was present after 3 h irradiation (Figure 5A); this indicates that light is promoting the loss of both chlorines from transplatin. Peaks 1−9 are all in the region for $H_3{}^{15}N$−Pt^{II} *trans* to N or Cl; however, the weak peak 10 is in the region for $H_3{}^{15}N$−Pt^{II} *trans* to O.[24] Therefore, photoisomer-

Table 4. ^{1}H and ^{15}N NMR Chemical Shifts and Assignments of the Peaks Produced on Reaction of Transplatin with 5′-GMP upon Irradiation and in the Dark

peaks[a]	$\delta(^{1}H)$ (ppm)	$\delta(^{15}N)$ (ppm)	assignments
1	3.60	−67.00	transplatin
2	3.94	−62.93	trans-[Pt($^{15}NH_3$)$_2$Cl(5-GMP)]
3	3.97	−61.06	trans-[Pt($^{15}NH_3$)$_2$(OH_2)(5-GMP)]
4	3.82	−64.36	-
5	4.20	−59.74	trans-[Pt($^{15}NH_3$)$_2$(5-GMP)$_2$]
6	3.65	−65.48	-
7	3.79	−66.13	-
8	3.73	−67.98	-
9	4.14	−58.27	-
10	4.26	−84.16	$H_3{}^{15}N$-Pt^{II} *trans* to oxygen

[a] See Figure 5 for peak labels.

ization or NH_3 substitution must also be occurring as has previously been observed for Pt^{II} complexes.[25] We also studied photoactivation of a sample containing complex **1** and 5′-GMP in a 1:2 ratio by 2D [^{1}H,^{15}N] NMR spectroscopy. To investigate whether irradiation influences the course of reactions with other cellular nucleophiles, we studied the reaction of ^{15}N-transplatin (1 mM) with 5′-GMP in the presence of glutathione (GSH) in a 1:2:3 molar ratio (pH 7.25). It was evident that 3 h of UVA irradiation significantly affected the course of the reaction. The 2D [^{1}H,^{15}N] HSQC NMR spectrum of the sample kept in the dark showed two major peaks at 3.71/−63.89 ppm (accounting for 40% of the total peaks by integration) and 4.10/−68.03 ppm (60%) perhaps assignable to 1:1 Pt:SG and 1:2 Pt:SG adducts.[26] After 3 h, UVA irradiation of these peaks accounted for only 42% (30% + 12%) of the observed signal intensity, and new cross-peaks at 4.05/−59.26, 3.99/−61.16, 3.89/−63.79, 3.81/−62.68, and 3.82/−61.49 ppm appeared in the spectrum, perhaps assignable to 5′-GMP and 5′-GMP/SG adducts.

Conclusions

Previous photochemical studies on cisplatin, transplatin, and [Pt(en)Cl_2] have reported that irradiation induces substitution of chloride ligands by solvent molecules, either H_2O or DMSO.[27,28] The results of the present work confirm this finding. The NMR spectroscopy results demonstrate that chloride ligands of transplatin are lost upon irradiation. The bis-GMP adduct *trans*-[Pt($^{15}NH_3$)$_2$(5′-GMP)$_2$]$^{2+}$ is observed for the irradiated sample but not for the sample kept in the dark, confirming higher efficiency of irradiated transplatin to form bifunctional adducts.

Monofunctional adducts of transplatin are formed on DNA relatively rapidly; however, their progression into bifunctional adducts is rarely observed. The lack of ability of transplatin to readily form bifunctional CLs is believed to be the reason for its lack of anticancer activity.[29,30] As UVA light promotes the loss of the second chloride of transplatin, we demonstrate that upon irradiation, bifunctional (mainly interstrand) CLs and DNA−protein CLs are produced with a considerably higher frequency than in the dark. DNA is the major pharmacological target of platinum compounds, and various bifunctional adducts are believed to be candidates for genotoxic lesions responsible for antitumor effects of bifunctional platinum compounds.

Modification by irradiated transplatin produces alterations in DNA that are different from those produced by transplatin in the dark. One possible explanation for this behavior is that the enhanced cytotoxicity of irradiated transplatin in comparison with nonirradiated transplatin could be a simple consequence of more extensive and/or distinct global perturbations of DNA due to interstrand CLs and DNA−protein CLs favorable for an antitumor effect. In this way, both the enhanced efficiency of formation and structural impact caused by the CLs formed by UVA-irradiated transplatin observed in this study may contribute to the biological behavior of irradiated transplatin and structurally related platinum drugs. The results of the present work also show for the first time that simple combination of an inactive platinum compound with UVA irradiation alters the DNA binding site of this compound into a DNA adduct of an active drug. Hence, the observations described in the present work might be useful when designing novel approaches to chemotherapy of tumors by platinum drugs. Further studies of the details of the alterations induced in DNA by irradiated transplatin including their recognition by damaged-DNA binding proteins, and repair, are therefore warranted.

Experimental Section

Starting Materials. Transplatin was obtained from Sigma (Prague, Czech Republic). The stock solution of transplatin was prepared at the concentration of 5×10^{-4} M in 10 mM $NaClO_4$ and stored at 4 °C in the dark. CT DNA (42% G+C, mean molecular mass ca. 20 000 kDa) was also prepared and characterized as described previously.[31] Plasmid pSP73 (2464 bp) was isolated according to standard procedures. The synthetic oligodeoxyribonucleotides were purchased from VBC-Genomics (Vienna, Austria) and purified as described previously;[32,33] in the present work their molar concentrations are related to the duplexes. Restriction endonuclease *Eco*RI and T4 polynucleotide kinase were purchased from New England Biolabs, and NF-κB (p50 dimer) from Active Motif (Rixensart, Belgium). Histone H1 from Roche Diagnostics, GmbH (Mannheim, Germany) acrylamide, bis(acrylamide), NaCN, TU, and urea from Merck KGaA (Darmstadt, Germany), SDS from Serva (Heidelberg, Germany), [γ-^{32}P]ATP and [α-^{32}P]dATP from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and proteinase K from Boehringer (Mannheim, Germany). Cell culture chemicals were obtained from Sigma (Poole, UK) and sterile disposable plastics from Greiner Bio-One (Cambridge, UK). For cell experiments, cisplatin and transplatin were prepared immediately before use in prewarmed (37 °C) Earle's Balanced Salt Solution (EBSS). Dissolution was facilitated by brief sonication and vortexing. 5′-GMP was purchased as the disodium salt from Acros Organics.

Syntheses. Transplatin for cell experiments was synthesized by an adaptation of the method of Kauffman.[34] ^{15}N-Transplatin was synthesized as previously described.[5]

Platination Reactions. CT DNA, plasmid pSP73, and oligodeoxyribonucleotide duplex were incubated with transplatin in 10 mM $NaClO_4$ at 37 °C in the dark or under irradiation by UVA light. The reaction of transplatin with DNA was terminated by quick cooling at −20 °C and adjusting the concentration of NaCl to 1.0 M. DNA samples were then dialyzed or precipitated by ethanol, and the r_b values were determined by FAAS.

Irradiation. The cells were irradiated by a bank of 2 × 6 ft Cosmolux RA Plus (Cosmedico) 15500/100 W light sources (5 J cm^{-2}: λ_{max} 365 nm), each filtered to attenuate UVB/UVC wavelengths. Irradiance was measured with a Waldmann PUVA meter, calibrated to the source using a double grating spectroradiometer (Bentham, UK). DNA samples were irradiated using the LZC-4V illuminator (photoreactor) (Luzchem, Canada) with temperature controller and with UVA tubes (4.3 mW cm^{-2}: λ_{max} 365 nm). NMR samples were irradiated with a UV lamp (2 × 15 W tubes, model VL-215L; Merck Eurolab, Poole, UK) which operates at 365 nm (1.5 mW cm^{-2}).

Cell Experiments. Human HaCaT (human adult low calcium high temperature) keratinocytes were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% nonessential amino acids. HaCaTs were a kind gift to the Photobiology Unit from Professor N. Fusenig (Heidleberg, Germany). A2780 and A2780CIS ovarian carcinoma cells were obtained from the European Collection of Animal Cell Cultures and maintained in RPMI supplemented with 10% FBS in the absence of antibiotics. Cells were grown in a humidified

atmosphere of 5% CO_2/95% air at 37 °C in the absence of antibiotics. Cultures were screened for mycoplasma contamination. Test compounds were prepared in EBSS immediately before use. For experiments, monolayers were washed in phosphate-buffered saline (PBS) and then incubated for 1 h at 37 °C in EBSS containing the test compound. After this time, control cells were treated identically to that of the test cells and sham irradiated.

Phototoxicity was monitored by measuring the uptake of neutral red dye into viable cells.[35] The dye was solubilized and absorbance was read at 540 nm. The IC_{50} value was defined as the concentration of compound that inhibited dye uptake by 50% relative to the control and was calculated using nonlinear regression (Graphpad Prism). Goodness of fit was determined from the R^2 values of the curves and 95% confidence intervals. Chlorpromazine (CPZ) was used as the positive control.

The effect of irradiated transplatin on nuclear DNA was measured in HaCaT cells using the single cell gel electrophoresis assay.[36] After irradiation, cells were treated with 50 μM H_2O_2 (4 °C, 5 min) and immediately processed for the comet assay.[5,37] Nuclei were stained with ethidium bromide and visualized using fluorescence microscopy (Nikon, E600). Fifty randomly selected nuclei from each slide were processed using image analysis (Komet, v.5.5, Andor Technology, UK). Slides were coded, and blinded analysis was performed. The parameter used to assess DNA migration was the percent of DNA in the comet tail. Experiments were performed three times in duplicate, and data points represent mean values ± SE for each test condition. Where applicable statistics were performed by ANOVA followed by Dunnett's test for multiple comparisons, or Student's t-test.

DNA Interstrand Cross-Linking. The amounts of interstrand CLs formed by transplatin in linear DNA were measured in pSP73 plasmid (2464 base pairs) which was first linearized by *Eco*RI (*Eco*RI cuts only once within pSP73 plasmid), 3′-end labeled by means of a Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP, and subsequently modified by transplatin. Linearized DNA (1 μg) was incubated with transplatin at $r_i = 5 \times 10^{-4}$ in the dark or UVA irradiated. The aliquots were withdrawn at various time intervals, the samples were precipitated by ethanol to remove unbound platinum, and the pellet was dissolved in 18 μL of a solution containing 30 mM NaOH, 1 mM EDTA, 6.6% sucrose, and 0.04% bromophenol blue. The samples were analyzed for the interstrand CLs by agarose gel electrophoresis under denaturing conditions (on alkaline 1% agarose gel).[12,38] After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and the interstrand cross-linked duplex were quantified by means of a Phosphor Imager (Fuji BAS 2500 system with AIDA software). The frequency of interstrand CLs (the amount of interstrand CLs per one molecule of transplatin bound to DNA multiplied by 100) was calculated using the Poisson distribution from the fraction of non-cross-linked DNA in combination with the r_b values and the fragment size. Other details of this assay can be found in previously published papers.[12,38,39]

Ternary DNA−Protein Reactions. The 23-bp duplex (for its nucleotide sequence, see Figure 3C, the central part of this sequence corresponded to DNA consensus sequence of NF-κB) was 5′-end-labeled at its top strand and globally modified by transplatin at r_b which corresponded to 1 platinum atom bound per duplex. Platinated DNA duplex (10 nM) was incubated with histone H1 or NF-κB (the molar ratio protein/duplex was 1:5) at 37 °C for 5 h in the appropriate buffer: 10 mM Tris·HCl, pH 7.9 + 20 mM NaCl (reactions with histone H1) or 42 mM HEPES, 42 mM KCl, 1 mM $MgCl_2$, 0.02 mM EDTA, 2,5% glycerol, 2% Ficoll (reactions with NF-κB). The platinated duplex was incubated with the protein for 5 h, and during this incubation the sample was irradiated with UVA or sham irradiated. The reactions were analyzed by native gel electrophoresis (5%/10% SDS-PAGE, samples were loaded in cracking buffer without dithiothreitol). Quantification of free and adducted DNA was determined with Phosphor Imager and AIDA software (Advanced Image Data Analyzer) purchased from Raytest (Isotopenmessgeräte GmbH, Germany).

NMR Spectroscopy. 1D ^{1}H and 2D [^{1}H,^{15}N] HSQC NMR spectra were recorded on a Bruker DMX500 NMR spectrometer (^{1}H: 500.13 MHz, ^{15}N: 50.7 MHz) using dioxane (3.764 ppm) as the internal $\delta(^1H)$ standard. All $\delta(^{15}N)$ values were referenced externally to $^{15}NH_4^+$ at $\delta = 0$ ppm. Spectra were acquired at 298 K and processed using Xwinnmr (2.0, Bruker UK Limited) software. pH values were measured with an Orion 710A pH meter equipped with a microcombination electrode (Aldrich) calibrated with Aldrich standard buffers (pH 4, 7, and 10) and were adjusted with $HClO_4$ (0.1 M). Samples were prepared in 90% H_2O/10% D_2O. The reaction between ^{15}N-transplatin (1 mM) and 5′-GMP was carried out in 1:1 and 1:2 molar ratios and in the presence of glutathione in 50 mM phosphate buffer (pH 7.25) with a ^{15}N-transplatin (1 mM):5′-GMP:gluathione molar ratio of 1:2:3.

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