

Transcription Inhibition by Platinum–DNA Cross-Links in Live Mammalian Cells

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Abstract: We have investigated the processing of site-specific Pt–DNA cross-links in live mammalian cells to enhance our understanding of the mechanism of action of platinum-based anticancer drugs. The activity of platinum drugs against cancer is mediated by a combination of processes including cell entry, drug activation, DNA-binding, and transcription inhibition. These drugs bind nuclear DNA to form Pt–DNA cross-links, which arrest key cellular functions, including transcription, and trigger a variety of responses, such as repair. Mechanistic investigations into the processing of specific Pt–DNA cross-links are critical for understanding the effects of platinum–DNA damage, but conventional *in vitro* techniques do not adequately account for the complex and intricate environment within a live cell. With this limitation in mind, we developed a strategy to study platinum cross-links on plasmid DNAs transfected into live mammalian cells based on luciferase reporter vectors containing defined platinum–DNA lesions that are either globally or site-specifically incorporated. Using cells with either competent or deficient nucleotide excision repair systems, we demonstrate that Pt–DNA cross-links impede transcription by blocking passage of the RNA polymerase complex and that nucleotide excision repair can remove the block and restore transcription. Results are presented for ~3800-base pair plasmids that are either globally platinated or carry a single 1,2-d(GpG) or 1,3-d(GpTpG) intrastrand cross-link formed by either *cis*-{Pt(NH₃)₂}²⁺ or *cis*-{Pt(*R,R*-dach)}²⁺, where {Pt(NH₃)₂}²⁺ is the platinum unit conveyed by cisplatin and carboplatin and *R,R*-dach is the oxaliplatin ligand, *R,R*-1,2-diaminocyclohexane.

Introduction

FDA-approved platinum-based anticancer drugs cisplatin, carboplatin, and oxaliplatin (Figure 1) are among the most effective chemotherapies in clinical application today. They are widely used to treat a variety of malignancies including colorectal, non-small-cell lung, and genitourinary cancers.^{1–3} The cytotoxic action of these compounds requires a combination of processes including cell entry, drug activation, DNA binding, and cellular responses.^{4–6} Upon cell entry, the platinum drugs are activated by aquation and bind nuclear DNA, giving rise to a limited number of adduct types. Cisplatin, in particular, forms predominantly 1,2-d(G*pG*)-Pt (65%) and 1,2-d(A*pG*)-Pt (25%) intrastrand cross-links and to a lesser extent 1,3-d(G*pNpG*)-Pt intrastrand (5–10%) and interstrand (<5%) adducts, where asterisks denote the platinated bases.^{4,5} Formation of platinated DNA lesions is an important determinant of the anticancer activity of platinum-based drugs, leading to arrest of key cellular functions and triggering a variety of responses.⁶ Understanding how the cell processes these Pt–DNA lesions is important for elucidating the mechanism of action of the

platinum drugs and forming a basis for the rational design of improved versions.

One activity that is important for platinum drug-mediated cytotoxicity is the arrest of RNA synthesis by Pt–DNA cross-links.⁷ Plasmids expressing the β -galactosidase reporter gene containing platinum–DNA adducts formed by cisplatin inhibit transcription 2–3-fold more effectively than adducts formed by *trans*-DDP, the inactive isomer of cisplatin (Figure 1).⁸ Because platinum drugs form a variety of DNA adducts, however, it is desirable to develop probes containing a single, site-specific Pt–DNA cross-link in order to investigate the specific roles of each in mediating biological activity. By introducing site-specifically platinated probes into cell-free extracts derived from mammalian cells, we previously elucidated the nature of RNA pol II arrest at the site of the platinated DNA lesion.⁹ Apart from transcription, cisplatin–DNA damage affects nucleotide excision repair (NER), an important pathway for removing the lesions.^{10,11} NER-defective human fibroblast cells derived from xeroderma pigmentosum (XP) patients are hyper-

- (1) Kelland, L. *Nat. Rev. Cancer* **2007**, *7*, 573–584.
- (2) Horwich, A.; Shipley, J.; Huddart, R. *Lancet* **2006**, *367*, 754–765.
- (3) Keys, H. M.; Bundy, B. N.; Stehman, F. B.; Muderspach, L. I.; Chafe, W. E.; Suggs, C. L.; Walker, J. L.; Gersell, D. N. *Engl. J. Med* **1999**, *340*, 1154–1161.
- (4) Jung, Y.; Lippard, S. J. *Chem. Rev.* **2007**, *107*, 1387–1407.
- (5) Jamieson, E. R.; Lippard, S. J. *Chem. Rev.* **1999**, *99*, 2467–2498.
- (6) Wang, D.; Lippard, S. J. *Nat. Rev. Drug Discovery* **2005**, *4*, 307–320.

- (7) Todd, R. C.; Lippard, S. J. *Metallomics* **2009**, *1*, 280–291.
- (8) Mello, J. A.; Lippard, S. J.; Essigmann, J. M. *Biochemistry* **1995**, *34*, 14783–14791.
- (9) Jung, Y.; Lippard, S. J. *J. Biol. Chem.* **2006**, *281*, 1361–1370.
- (10) Husain, I.; Chaney, S. G.; Sancar, A. *J. Bacteriol.* **1985**, *163*, 817–823.
- (11) Zamble, D. B.; Mu, D.; Reardon, J. T.; Sancar, A.; Lippard, S. J. *Biochemistry* **1996**, *35*, 10004–10013.

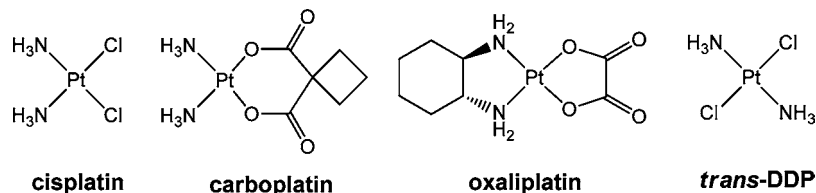


Figure 1. Chemical structures of platinum anticancer drugs and the inactive cisplatin isomer, *trans*-diamminedichloroplatinum(II).

sensitive to platinum drugs.¹² These cells contain specific defects in one of seven XP genes, XPA through XPG, and are generally more sensitive to treatment by platinum drugs compared to normal human fibroblasts. Because the nature of their genetic defect is well characterized, XP cells provide excellent models to study the effects of platinum drugs in an NER-deficient environment.

Until now, mechanistic investigations of platinum–DNA damage processing have largely been carried out *in vitro* using site-specifically platinated probes in conjunction with purified enzymes or cell-free extracts.^{13–19} These systems have been quite informative, but they do not adequately replicate the conditions within a live mammalian cell. In order to extend our investigations to a more accurate albeit complex environment, we devised a strategy to study transcription inhibition by Pt–DNA lesions that is based on expression vectors containing single site-specific platinated DNAs as probes. We sought to determine both the extent to which Pt–DNA cross-links can inhibit transcription by impeding the passage of RNA polymerase (pol) II in live cells and the effect of NER in removing the blockage and restoring transcription. To test the methodology, we first prepared globally platinated transcription probes before moving to the synthetically more challenging site-specific constructs. The results are described and discussed in this report.

Results

Methodology. Our methodology is based on the use of platinated DNA plasmids constructed from a recombinant mammalian expression vector containing a reporter gene under the control of a promoter for subsequent transfection into mammalian cells as functional transcription probes. Introduction of Pt–DNA cross-links into the plasmids was achieved site-specifically using oligonucleotide insertion. Expression of the reporter gene, unimpeded in the absence of the lesion, is adversely affected if the lesion is an effective inhibitor of transcription. By determining transcription levels under specific cellular conditions, it becomes possible to determine how mammalian cells process specific Pt–DNA cross-links.

The design and construction of the parent vector, pGLuc, which contains *Gaussia* luciferase (GLuc) as the reporter gene

under the control of CMV promoter, have been described.²⁰ The GLuc enzyme is an ideal reporter because it requires no post-translational modification, thus allowing rapid and responsive determination of intracellular transcription levels. Enzymatic activity can also be quantified to a high degree of sensitivity by bioluminescence assays using coelenterazine as the substrate. In addition, GLuc is naturally secreted from the cell, so determination of expression levels can be performed by sampling cell culture media rather than having to prepare cell extracts, as with conventional luciferase assays.^{21,22} Transfection of transcription probes into mammalian cells was carried out using liposomal transfecting agents. To yield meaningful data, experiments were conducted using a cell line pair comprising a parental line and its genetic variant. This approach allowed us to study transcription profiles under conditions where specific gene expression was either turned on or off, using the parental cell line as the control. XPF human fibroblast cells (GM08437, Coriell Institute cell depository) were used as an NER-deficient cell model. To generate the NER-competent complement, XPF cells were stably transfected with XPF cDNA to generate XPF-corrected XPF cells,²³ designated XPF_{corr}.

Preparation of Transcription Probes. Globally platinated transcription probes were prepared by reacting pGLuc with varying concentrations of cisplatin in buffer.^{24,25} Platination levels, expressed as the ratio of bound platinum per plasmid (Pt/plasmid), were determined by measuring the Pt content of the plasmid solution by atomic absorption spectroscopy and dividing it by the DNA concentration. A set of six plasmids with added Pt/plasmid ratios ranging from 0 to 65.0 were prepared corresponding to r_b values, defined as platinum bound per nucleotide, from 0 to 8.6×10^{-3} . Transcription experiments were performed by monitoring *Gaussia*-converted GLuc levels as a function of Pt/plasmid ratio in XPF and XPF_{corr} cells at 8 h intervals over a period of 48 h.

Site-specifically platinated probes were prepared from pGLuc-derived plasmids by creating a short single-stranded gap, followed by filling-in of the gap with a platinated oligonucleotide (Figure 2). The detailed synthetic methodology was reported previously.^{20,26,27} pGLuc was modified with a 30-bp sequence between CMV and GLuc to prepare vectors that can accom-

- (12) Plooy, A. C. M.; van Dijk, M.; Berends, F.; Lohman, P. H. M. *Cancer Res.* **1985**, *45*, 4178–4184.
- (13) Moggs, J. G.; Yarema, K. J.; Essigmann, J. M.; Wood, R. D. *J. Biol. Chem.* **1996**, *271*, 7177–7186.
- (14) Moggs, J. G.; Szymkowski, D. E.; Yamada, M.; Karran, P.; Wood, R. D. *Nucleic Acids Res.* **1997**, *25*, 480–490.
- (15) Cohen, S. M.; Jamieson, E. R.; Lippard, S. J. *Biochemistry* **2000**, *39*, 8259–8265.
- (16) Wei, M.; Cohen, S. M.; Silverman, A. P.; Lippard, S. J. *J. Biol. Chem.* **2001**, *276*, 38774–38780.
- (17) Jung, Y.; Mikata, Y.; Lippard, S. J. *J. Biol. Chem.* **2001**, *276*, 43589–43596.
- (18) Shivji, M. K. K.; Moggs, J. G.; Kuraoka, I.; Wood, R. D. *Methods Mol. Biol.* **2006**, *314*, 435–456.
- (19) Malina, J.; Novakova, O.; Vojtiskova, M.; Natile, G.; Brabec, V. *Biophys. J.* **2007**, *93*, 3950–3962.

- (20) Ang, W. H.; Lippard, S. J. *Chem. Commun.* **2009**, 5820–5822.
- (21) Tannous, B. A.; Kim, D.-E.; Fernandez, J. L.; Weissleder, R.; Breakefield, X. O. *Mol. Ther.* **2005**, *11*, 435–443.
- (22) Michelini, E.; Cevenini, L.; Mezzanotte, L.; Ablamsky, D.; Southworth, T.; Branchini, B. R.; Roda, A. *Photochem. Photobiol. Sci.* **2008**, *7*, 212–217.
- (23) Chen, Z.; Xu, X. S.; Harrison, J.; Wang, G. *Biochem. J.* **2004**, *379*, 71–78.
- (24) Todd, R. C.; Lovejoy, K. S.; Lippard, S. J. *J. Am. Chem. Soc.* **2007**, *129*, 6370–6371.
- (25) Lovejoy, K. S.; Todd, R. C.; Zhang, S.; McCormick, M. S.; D’Aquino, J. A.; Reardon, J. T.; Sancar, A.; Giacomini, K. M.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 8902–8907.
- (26) Ang, W. H.; Brown, W. W.; Lippard, S. J. *Bioconjugate Chem.* **2009**, *20*, 1058–1063.
- (27) Wang, H.; Hays, J. B. *Mol. Biotechnol.* **2001**, *19*, 133–140.

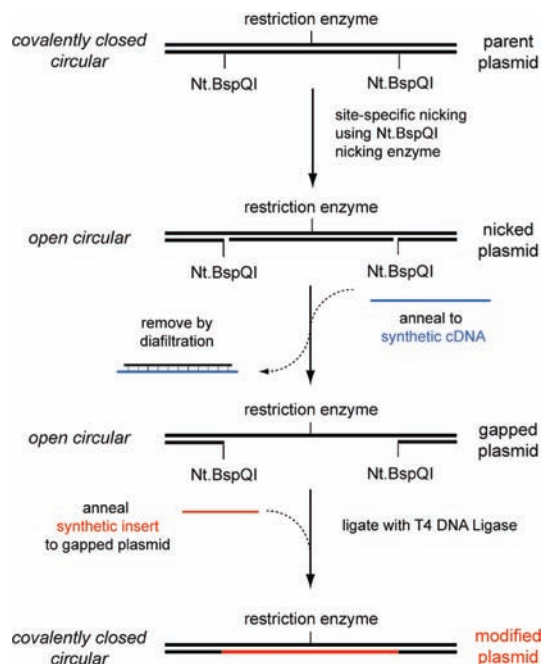


Figure 2. Scheme for preparing site-specifically platinated probes.

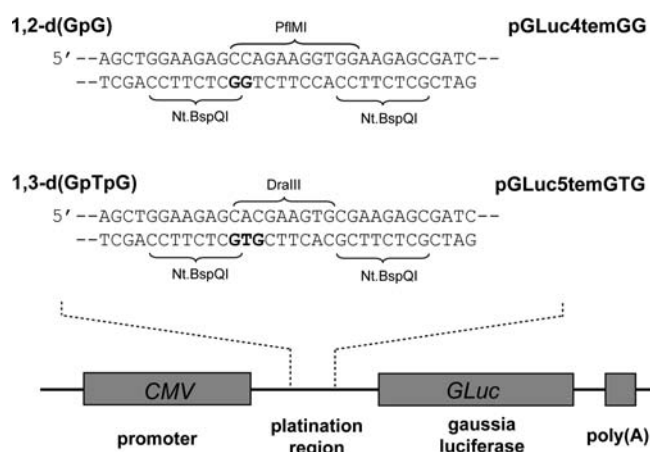


Figure 3. Site-specifically platinated probes containing Pt–DNA cross-links of cisplatin and oxaliplatin; platinations sites are highlighted in bold.

moderate the oligonucleotide insertions. The sequence was designed such that there were two recognition sites for Nt.BspQI nicking enzymes to create site-specific cuts on the template strand 16 nucleotides apart, with the intended platinations site positioned between the two nicking positions. Two plasmids designed to accommodate 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt adducts were prepared and designated as pGLuc4temGG and pGLuc5temGTG, respectively (Figure 3). Detailed experimental protocols for creating single-stranded gaps and inserting the platinated oligonucleotides into these plasmids are described in the Supporting Information. Vectors pGLuc4temGG and pGLuc5temGTG containing *cis*-{Pt(NH₃)₂}-DNA and {Pt(dach)}-DNA cross-links were prepared, plasmids corresponding to lesions formed by cisplatin and oxaliplatin, respectively (Table 1), where dach is the *R,R*-1,2-diaminocyclohexane ligand in oxaliplatin. Unplatinated oligonucleotides were also inserted to generate control plasmids.

Transcription Profiling of Globally Platinated Probes. The effects of platinum–DNA damage on transcription were inves-

Table 1. Designation of Site-Specifically Platinated Plasmids

type of lesion	1,2-d(G*pG*)-Pt	1,3-d(G*pTpG*)-Pt
plasmid designation	pGLuc4temGG	pGLuc5temGTG
unplatinated control	+is	+is
cisplatin adduct	+is-PtA ₂	+is-PtA ₂
<i>cis</i> -{Pt(NH ₃) ₂ }		
oxaliplatin adduct	+is-Pt(dach)	+is-Pt(dach)
{Pt(dach)}		

tigated using pGLuc plasmids that were globally platinated with cisplatin. In previous reports, we demonstrated, using other reporter constructs, that Pt–DNA cross-links formed by cisplatin are strong inhibitors of transcription.^{8,25} However, in those assays, transcription levels were assayed at a single time point because cell lysis was required to release the reporter enzyme for analysis. With the use of GLuc as the reporter enzyme, analyses could be performed at regular time intervals on a given sample because the enzyme is extruded into the cell media. Synchronized XPF and XPF_{corr} cells were transfected with the same set of platinated probes, and the cell media were collected at 8 h intervals. The cell media samples thus obtained were analyzed for GLuc activity and normalized against unplatinated controls. Since total cell media were collected and replaced with fresh media, total transcription levels within the 8 h interval could be quantitated.

Transcription profiles of globally platinated probes in XPF and XPF_{corr} cells were obtained by plotting normalized GLuc expression levels against platinations levels (Pt/plasmid ratio) as a function of time (Figure 4). The normalized transcription levels followed an exponential relationship with respect to platinations levels for both XPF and XPF_{corr} cells at 8 h following transfection (Figure 5), the first time point at which cell media were collected. In subsequent assays, recovery of transcription was observed for both XPF and XPF_{corr} cells, with a pronounced change in profile for the latter. Transcription levels from probes containing low platinations levels (Pt/plasmid < 10) were restored to >90% of control values in XPF_{corr} cells, whereas transcription levels in XPF cells remain suppressed.

In order to compare transcription inhibition between the two cell lines quantitatively, the data were fit to an exponential curve, and a *D*₀ value, defined as the number of Pt lesions per plasmid required to reduce transcription levels to 37% of the control,⁸ was extracted from the fitted curves (Figure 5). The *D*₀ values 8 h after transfection for XPF and XPF_{corr} cells were similar, 4.0 and 5.3, respectively, which is not surprising since XPF and XPF_{corr} have the same genotypic origin and would have processed the probes in a similar manner. Upon further sampling, however, it was evident that recovery of transcription in XPF_{corr} was superior to that in the XPF cells. At the later time points, the transcription profile could no longer be adequately modeled using an exponential curve, and the *D*₀ values obtained represented lower limits. The *D*₀ values in XPF and XPF_{corr} are listed in Table 2. At 48 h, *D*₀ values for XPF and XPF_{corr} cells were approximately 10.2 and 20.7. The difference in *D*₀ values across the time intervals and the difference in transcription profile for the same probes in two different cell lines indicate a role for NER in the restoration of transcription.

Transcription Inhibition by Site-Specific Pt–DNA Cross-Links.

In order to investigate how specific Pt–DNA adducts inhibit transcription, we investigated site-specifically platinated probes containing the major DNA adducts of cisplatin and oxaliplatin, namely 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt cross-links.

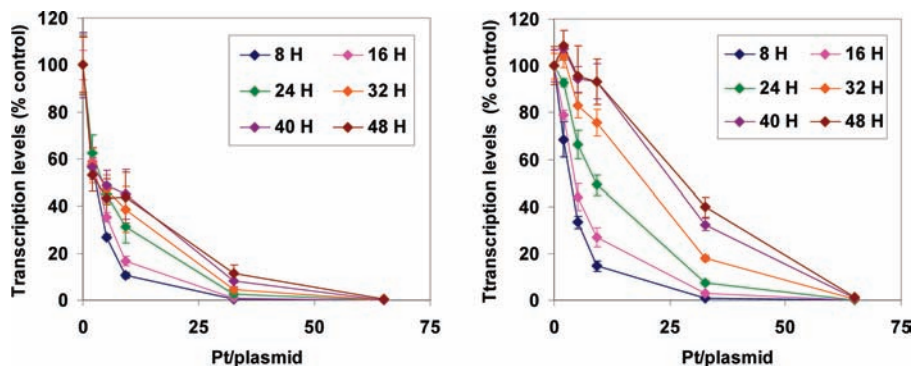


Figure 4. Transcription profiles of globally platinated probes in XPF (left) and XPF_{corr} (right) cells.

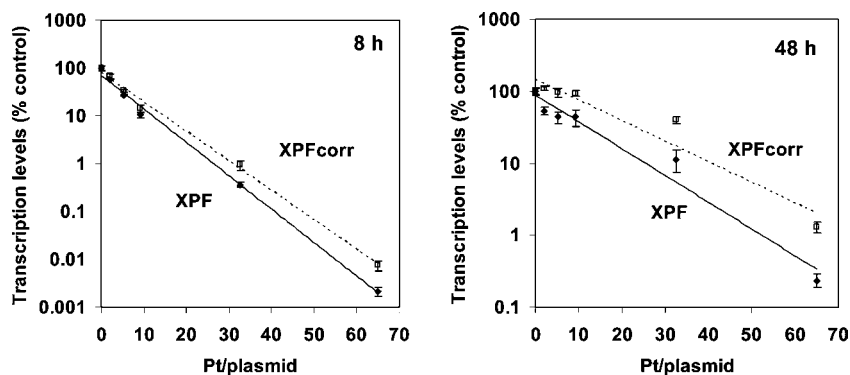


Figure 5. Comparison of transcription profiles of globally platinated probes in XPF and XPF_{corr} cells 8 h (left) or 48 h (right) after transfection.

Table 2. D_0 Values of Globally Platinated Probes Assayed at Different Time Intervals after Transfection^a

time after transfection	XPF (Pt/plasmid)	XPF _{corr} (Pt/plasmid)
8 h	4.0	5.4
16 h	5.3	7.9
24 h	7.6	11.5
32 h	8.7	15.4
40 h	10.0	18.7
48 h	10.2	20.7

^a D_0 value is defined as the number of Pt lesions per plasmid required to reduce transcription levels to 37% of the control.

Instead of introducing platinated lesions randomly, a single platinated DNA lesion was inserted on the template strand between the promoter and reporter gene sequences to create a block to RNA pol II prior to transcribing the GLuc reporter gene. The lesion was positioned about 50 bp downstream from the transcription start site, so that the transcription complex would encounter the lesion during mRNA elongation. In keeping with experimental data *in vitro*, we anticipated that transcription would stall at the lesion site, resulting in a decrease in GLuc expression levels versus unplatinated controls. We also expected GLuc expression levels of the platinated probes to be lower in XPF as compared to XPF_{corr} cells, since XPF cells are incapable of removing the Pt–DNA blockage.

Transfections were carried out and the transcription levels determined after 24 h, corresponding to the approximate time for a complete mammalian cell cycle. In XPF cells, transcription levels of probes containing 1,2-d(G*pG*)-PtA₂ and 1,2-d(G*pG*)-Pt(dach) were 50.0 ± 7.9% and 35.0 ± 3.4% of unplatinated controls, respectively, whereas in XPF_{corr}, the corresponding levels were 96.6 ± 12.7% and 63.4 ± 4.5%. For 1,3-d(G*pTpG*)-PtA₂ and 1,3-d(G*pTpG*)-Pt(dach), the levels

were 37.7 ± 6.7% and 23.7 ± 3.2%, respectively, for XPF cells and 73.2 ± 9.2% and 51.3 ± 10.2%, respectively, for XPF_{corr} cells (Figure 6). The higher transcription levels in XPF_{corr} compared with XPF cells indicate that the NER-competent cells are better able to overcome the Pt–DNA transcriptional block.

Discussion

Pt–DNA Cross-Links Inhibit RNA Transcription in Live Cells by Blocking RNA Elongation. Pt–DNA cross-links have previously been shown to be good inhibitors of transcription *in vitro* using both cell-based assays and reconstituted systems,^{8,9,28–30} an observation reproduced by the present live cell luciferase assays. There are several hypotheses about the mechanism of transcription inhibition by DNA adducts of platinum anticancer drugs.⁷ One hypothesis supported by compelling structural and biochemical evidence is that Pt–DNA cross-links present a physical impediment, or roadblock, to transcription during RNA elongation and result in stalling of the RNA pol II enzyme at the lesion site.^{9,28,31} Since transcription from the globally platinated pGLuc probes depends on accessibility of the promoter and reporter gene sequences to RNA pol II, we expected that there would be a greater probability for the enzyme to encounter Pt lesions on probes having higher platination levels. The data obtained indicate a strong dependence of transcription levels on Pt/plasmid ratios in both cell lines, consistent with this hypothesis (Figure 5).

(28) Tremeau-Bravard, A.; Riedl, T.; Egly, J.-M.; Dahmus, M. E. *J. Biol. Chem.* **2004**, *279*, 7751–7759.

(29) Cullinane, C.; Mazur, S. J.; Essigmann, J. M.; Phillips, D. R.; Bohr, V. A. *Biochemistry* **1999**, *38*, 6204–6212.

(30) Tomaletti, S.; Patrick, S. M.; Turchi, J. J.; Hanawalt, P. C. *J. Biol. Chem.* **2003**, *278*, 35791–35797.

(31) Damsma, G. E.; Alt, A.; Brueckner, F.; Carell, T.; Cramer, P. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1127–1133.

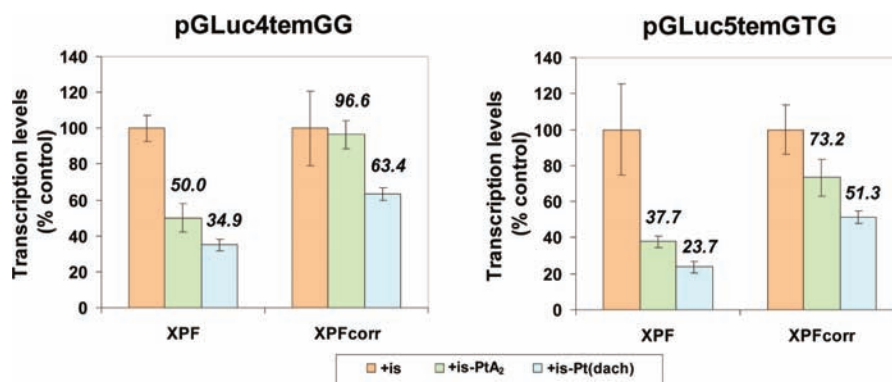


Figure 6. Transcription of site-specifically platinated probes containing 1,2-d(G*pG*)-Pt (pGLuc4temGG, left) and 1,3-d(G*pTpG*)-Pt (pGLuc5temGTG, right) cross-links 24 h after transfection.

To investigate whether a *single* platinated DNA lesion would block RNA elongation, we engineered a Pt–DNA cross-link site-specifically into the template strand of pGLuc between the promoter and the reporter gene sequence as a block to transcription. The cross-link was deliberately positioned 50 bp downstream of the transcription start site so that the pol II enzyme would encounter the lesion during the elongation rather than the initiation phase.^{32,33} The positioning of the cross-link upstream of the reporter gene was primarily to facilitate vector preparation, since insertion of the artificial 30-bp platination sequence into the reporter gene sequence could adversely affect the viability of the luciferase enzyme.

Our results reveal significant transcription inhibition using the site-specifically platinated probes in NER-deficient XPF cells 24 h after transfection in comparison to unplatinated controls. Thus, for the first time, we are able to demonstrate the ability of a defined adduct to block transcription in a live cell. To the extent that this activity reflects the sensitivity of cancer cells of different lineage to platinum–DNA lesions derived from various members of the cisplatin family of drugs, it offers the potential to personalize treatment options for future medical applications.

The results are consistent with our previous report employing run-off assays performed on immobilized site-specifically platinated DNA templates *in vitro* using HeLa nuclear extracts, which revealed that transcription is inhibited at the site of the platinated lesion.⁹ In addition, the GLuc expression data indicate lower levels of transcription from the 1,2-d(G*pG*)-Pt(dach) and 1,3-d(G*pTpG*)-Pt(dach) probes by comparison to 1,2-d(G*pG*)-PtA₂ and 1,3-d(G*pTpG*)-PtA₂, respectively, suggesting that Pt(dach)–DNA lesions are stronger transcription blocks than PtA₂–DNA adducts. This result supports the roadblock hypothesis because the bulky dach ligand presents a greater steric hindrance than the two ammine ligands. A similar observation was made using *in vitro* transcription on immobilized DNA templates containing the same Pt–DNA cross-links by T7 RNA pol enzyme using “transcription walking”. This experiment revealed that the enzyme completely arrests at the first G residue of the 1,2-d(G*pG*)-Pt(dach) and 1,3-d(G*pTpG*)-Pt(dach) lesions on the DNA template but is able to bypass the residue of 1,2-d(G*pG*)-PtA₂ and 1,3-d(G*pTpG*)-PtA₂ lesions, indicating more effective inhibition.³⁴ An important distinction between the present results, obtained in live cells,

compared to the earlier *in vitro* assay is the inability of the site-specific Pt adducts to form an absolute block to transcription in the XPF-corrected cells. Extension of such studies to cells of different genetic backgrounds and tissue type will be quite valuable for assessing the clinical potential of existing and new members of the platinum anticancer drug family.

In summary, the data from the present luciferase live cell assay using the site-specifically platinated probes support the hypothesis that platinated DNA adducts inhibit transcription in living cells by impeding RNA elongation. The results distinguish between non-leaving-group ligands on platinum, ammine vs *R,R*-1,2-dach, and between 1,2- vs 1,3-cross-links.

Nucleotide Excision Repair of Platinated DNA Adducts Restores Transcription. The presence of DNA lesions caused by mutagens of endogenous or exogenous origin can have deleterious effects on cells. To defend against these insults, the cell has evolved NER as a versatile and sophisticated mechanism to remove the damage.³⁵ Two sub-pathways, global genomic NER (GG-NER) and transcription-coupled NER (TC-NER), which differ in their mode of damage-recognition, can be evoked. In GG-NER, DNA damage is recognized by the XPC-hHR23B-Cen2 heterotrimeric complex, whereas in TC-NER the polymerase complex stalled at the site of a lesion is the trigger for repair.^{36–38} Repair of cisplatin damage is performed predominantly via the TC-NER pathway because cells with defective TC-NER-specific genes, e.g., Cockayne Syndrome B (CS-B), are more sensitive to cisplatin treatment than cells lacking GG-NER-specific genes, e.g., XPC.^{37,39} In addition, with the use of plasmids globally platinated with cisplatin in HeLa nuclear extracts, it was demonstrated that the arrest of transcription on cisplatin-damaged DNA induced RNA pol II ubiquitylation.⁴⁰ Since this ubiquitylation was absent in TC-NER-deficient CS cells, it was speculated that ubiquitylation is a flag to promote recruitment of NER machinery to the stalled

(32) Dahmus, M. E. *J. Biol. Chem.* **1996**, *271*, 19009–19012.

(33) Bartholomew, B.; Dahmus, M. E.; Meares, C. F. *J. Biol. Chem.* **1986**, *261*, 14226–14231.

(34) Jung, Y.; Lippard, S. J. *J. Biol. Chem.* **2003**, *278*, 52084–52092.

(35) de Laat, W. L.; Jaspers, N. G. J.; Hoeijmakers, J. H. J. *Genes Dev.* **1999**, *13*, 768–785.

(36) Iyer, N.; Reagan, M. S.; Wu, K.-J.; Canagarajah, B.; Friedberg, E. C. *Biochemistry* **1996**, *35*, 2157–2167.

(37) van Gool, A. J.; Citterio, E.; Rademakers, S.; van Os, R.; Vermeulen, W.; Constantinou, A.; Egly, J.-M.; Bootsma, D.; Hoeijmakers, J. H. J. *EMBO J.* **1997**, *16*, 5955–5965.

(38) Hoogstraten, D.; Bergink, S.; Ng, J. M. Y.; Verbiest, V. H.; Luijsterburg, M. S.; Geverts, B.; Raams, A.; Dinant, C.; Hoeijmakers, J. H. J.; Vermeulen, W.; Houtsmuller, A. B. *J. Cell Sci.* **2008**, *121*, 2850–2859.

(39) Furuta, T.; Ueda, T.; Aune, G.; Sarasin, A.; Kraemer, K. H.; Pommier, Y. *Cancer Res.* **2002**, *62*, 4899–4902.

(40) Lee, K.-B.; Wang, D.; Lippard, S. J.; Sharp, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4239–4244.

polymerase, thereby establishing a role for TC-NER in the processing of Pt–DNA cross-links.⁴¹

Since the removal of platinated lesions should lead to the resumption of transcription, we decided to investigate changes in the transcription profile of the globally platinated probes as a function of time. The XPF cell line is an excellent NER-deficient model because its genetic mutation leads to a defective XPF that is unable to stably heterodimerize with ERCC1 to form the XPF/ERCC1 endonuclease complex.⁴² The XPF/ERCC1 endonuclease is a critical NER component responsible for creating an incision 5' to the site of DNA damage; therefore, XPF cells are incapable of removing a platinated lesion from the damage site.⁴³ It was therefore not surprising that transcription recovery of globally platinated probes in XPF cells was slow during the 48 h period in which the experiment was conducted (Figure 4). In contrast, transcription on the same set of probes in NER-competent XPF_{corr} cells changed dramatically, with full recovery of transcription in the minimally platinated probes within 48 h of transfection.

To further investigate the cellular response to a platinated lesion positioned on the path of the RNA elongation complex, transcription levels from site-specifically platinated plasmids were compared in XPF and XPF_{corr} cells. After 24 h, transcription from the probes was significantly higher in XPF_{corr} than XPF cells, with nearly full recovery of transcription occurring in the probe containing 1,2-d(G*pG*)-PtA₂ cross-link (Figure 6). The data suggest that XPF_{corr} cells are able to more effectively overcome a platinated lesion to restore transcription than XPF cells, consistent with earlier findings. The data also indicate that Pt(dach) lesions not only are better inhibitors of transcription but are less efficiently repaired than PtA₂ adducts. These findings differ from those of previous work based on excision assays using site-specifically platinated linear DNA probes with HeLa cell-free extracts. In that report, fragments containing the platinated lesion excised from the probes, which were treated with the extracts for 60 min, were resolved by polyacrylamide gel electrophoresis and quantitated using radiography.⁴⁴ No significant difference between the 1,2-d(G*pG*)-Pt adducts of cisplatin and oxaliplatin was observed.

In our assays, repair efficiencies were inferred from transcription data using NER-proficient/deficient whole cell models. Although excision assays provide a more direct measurement of repair rates, our assays carried out in live mammalian cells provide a more realistic environment. Transfection of platinated plasmids into cell hosts other than HeLa may exhibit different degrees of transcription inhibition, however, and we do not rule out the possibility of greater blockage of RNA pol II in live cells than observed in the present study. The likelihood that cell type will affect the degree of transcription inhibition and the potential value of this information have already been discussed.

Repair of Pt–DNA Cross-Links by Other Mechanisms. Another subtle difference in the data obtained with the live cell luciferase assays described in this report and run-off assays using HeLa nuclear extracts reported previously relates to the extent

of observed transcription inhibition.^{9,28,30} In the run-off assays, transcription was almost completely inhibited by 1,2-d(G*pG*)-PtA₂ and 1,3-d(G*pTpG*)-PtA₂ cross-links, with very low levels of run-off products detected. By contrast, high residual transcription levels of 50.0% and 37.7% were detected for site-specifically platinated probes in repair-deficient XPF cells using probes containing 1,2-d(G*pG*)-PtA₂ and 1,3-d(G*pTpG*)-PtA₂ lesions, respectively. This result indicates that cells can bypass transcription even in the absence of functioning NER machinery. In addition, transcription of globally platinated probes recovered as a function of time in the NER-deficient XPF cells, albeit at a slower rate compared to that in XPF_{corr} cells, suggesting removal of the Pt blockage.

These observations point to the presence of other mechanisms besides NER for transcription past the site of Pt–DNA cross-linking. Examples of such mechanisms include processes that eliminate the platinum block during the long time course of the luciferase assay, such as mismatch repair and base excision repair.^{14,45,46} In vitro experiments using immobilized platinated DNA templates and cell extracts require short exposure times to prevent degradation of the DNA templates in the cell extracts. The present live cell luciferase experiments therefore suggest that, whereas NER is important for the repair of platinated DNA lesions, other mechanisms might also be present that are not adequately modeled by the conventional in vitro assays.

Conclusion

We report for the first time a system for investigating in live mammalian cells the processing of specific Pt–DNA cross-links formed by platinum anticancer drugs using transcriptionally active expression vectors containing the programmed adducts. Our results reveal how specific Pt–DNA cross-links inhibit transcription in live cells, to varying degrees depending on the type of lesion, by impeding the passage of RNA pol II, and we demonstrate that NER can effectively remove the blockage and restore transcription. The data also indicate that there are other repair mechanisms in operation, besides NER, that can repair Pt–DNA cross-links from the DNA template. By studying the processing of Pt–DNA cross-links within a realistic cellular environment, our investigations provide a major tool for elucidating the mechanism of action of clinically important platinum-based drugs and offer the potential to select a platinum compound for treatment of tumors on the basis of its ability to inhibit transcription from a globally or site-specifically modified probe in live cells derived from the cancer tissue.

Experimental Section

Materials and Methods. All chemical reagents were obtained from Sigma-Aldrich unless otherwise stated. Included are pCMV-GLuc (New England Biolabs), Lipofectamine 2000 and OptiMEM cell media (Invitrogen), fetal bovine serum (Hyclone), coelenterazine (Nanolight technologies), and DMEM cell media and G418 sulfate (MediaTech). XPF (GM08437) cells were obtained from the Coriell Cell Depositories (Coriell Institute, Camden, NJ). XPF-corrected XPF cells (XPF_{corr}), originally established by stably transfecting XPF cDNA into XPF cells, were generously donated by Dr. Gan Wang (Wayne State University, Detroit, MI). Cells were grown in a humidified incubator at 37 °C under 5% CO₂.

(41) Bregman, D. B.; Halaban, R.; van Gool, A. J.; Henning, K. A.; Friedberg, E. C.; Warren, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11586–11590.

(42) Matsumura, Y.; Nishigori, C.; Yagi, T.; Imamura, S.; Takebe, H. *Hum. Mol. Genet.* **1998**, *7*, 969–974.

(43) Ferry, K. V.; Hamilton, T. C.; Johnson, S. W. *Biochem. Pharmacol.* **2000**, *60*, 1305–1313.

(44) Reardon, J. T.; Vaisman, A.; Chaney, S. G.; Sancar, A. *Cancer Res.* **1999**, *59*, 3968–3971.

(45) Yamada, M.; O'Regan, E.; Brown, R.; Karran, P. *Nucleic Acids Res.* **1997**, *25*, 491–495.

(46) Yang, J.; Parsons, J.; Nicolay, N. H.; Caporali, S.; Harrington, C. F.; Singh, R.; Finch, D.; D'Atri, S.; Farmer, P. B.; Johnston, P. G.; McKenna, W. G.; Dianov, G.; Sharma, R. A. *Oncogene* **2010**, *29*, 463–468.

XPF cells were maintained in DMEM containing 4.5 g/L glucose supplemented with 10% FBS and antibiotics, and XPF_{corr} was maintained in DMEM containing 4.5 g/L glucose supplemented with 10% FBS, antibiotics, and 0.1 mg/mL G418 sulfate.

Quantification of Pt content on globally platinated plasmids was carried out by graphite furnace atomic absorption spectroscopy (GF-AAS) using the Perkin-Elmer AAnalyst 300 system. DNA concentrations were measured by UV/vis spectroscopy at 260 nm using a Varian Cary 1E-spectrometer fitted with a microprobe and an extinction coefficient of 50 $\mu\text{g}/\text{mL}/\text{OD}$. The r_b value is defined here as the number of atoms of platinum complex bound per nucleotide. For site-specifically platinated plasmids, DNA quantification was performed using the picogreen assay (Molecular Probes) against a plasmid DNA standard curve (0, 10, 20, 30, 40, 50 $\mu\text{g}/\text{mL}$) on the BioTek Synergy 2 fluorescence microplate reader ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 528 \text{ nm}$). Agarose gel electrophoreses (0.8–1.0% w/v) containing 0.5 $\mu\text{g}/\text{mL}$ EtdBr were imaged using the BioRad Fluor-S MultiImager.

Vector Construction and Preparation. A mammalian vector expressing Gaussia luciferase, pGLuc, was derived from the commercial pCMV-GLuc by PCR-mediated deletion, which was performed in order to remove restriction sites of *Bsp*QI and the SV40 origin of replication, as previously described.²⁰ This procedure renders the plasmid incompetent for replication in the host cells. pGLuc was treated sequentially with *Hind*III and *Bam*HI to digest the vector between the CMV promoter and GLuc reporter genes. It was then ligated with a synthetic insert containing sequences for 1,2-d(G*pG*)-Pt DNA lesion incorporation to yield pGLuc4temGG, as described in the Supporting Information. pGLuc5temGTG, designed to specifically incorporate a 1,3-d(G*pTpG*)-Pt adduct, was prepared from pGLuc in a similar fashion.

Preparation of Globally Platinated Plasmids. pGLuc plasmids (50 nM) were treated with cisplatin (0.5, 1, 2, 4, 8 μM) in buffer (24 mM HEPES pH 6.0, 10 mM NaCl) for 16 h at 25 °C. A mock platination reaction was also carried out as a control using water in place of cisplatin. Upon completion, the reaction mixtures were dialyzed against TE/NaCl buffer (10 mM Tris·HCl pH 8.0, 1 mM EDTA, 200 mM NaCl) for 4 h, followed by TE buffer (10 mM Tris·HCl pH 8.0, 1 mM EDTA) for 8 h with 2 exchanges of TE buffer solution. The r_b values of plasmids were determined to be 0, 2.8×10^{-4} , 6.9×10^{-4} , 1.2×10^{-3} , 4.3×10^{-3} , 8.6×10^{-3} , corresponding to 0, 2.1, 5.2, 9.2, 32.6, 65.0 Pt per plasmid.

Preparation of Site-Specifically Platinated Plasmids. Site-specifically platinated plasmids containing a single Pt–DNA lesion positioned between the CMV promoter and Gaussia luciferase reporter gene were prepared as described previously.²⁶ Experimental details are provided in the Supporting Information. Incorporation of 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt lesion-containing oligonucleotides was carried out on pGLuc4temGG and pGLuc5temGTG plasmids, respectively. Plasmids containing cisplatin-type *cis*-{Pt(NH₃)₂}–DNA adducts were designated with the “+is-PtA₂” insertion strand suffix, and plasmids with oxaliplatin-type {Pt(dach)}–DNA adducts were designated as “+is-Pt(dach)”. Control plasmids, prepared by mock insertion of unplatinated oligonucleotides, were designated with an “+is” suffix. A summary of plasmids used in this study is provided in Table 1.

Transient Transfection of Cells for Transcription Assays. Transcription assays were carried by transient transfection of the plasmids into XPF and XPF_{corr} cells, synchronized at G1/S phase, followed by measurement of the levels of luciferase reporter gene expression. Cell synchronization at G1/S was accomplished using a double thymidine block. Briefly, XPF and XPF_{corr} cells were

distributed on a 96-well plate at a density of 5000 cells per well. At 24 h after cell plating, cell media were aspirated, and fresh pre-warmed media containing 2.5 mM thymidine were added. After a 19 h incubation period, the cell media were replaced, and the cells were incubated for 12 h under thymidine-free conditions. The cell media were then aspirated, and fresh pre-warmed media containing 2.5 mM thymidine were added. The cells were incubated for an additional 12 h to achieve G1/S synchronization and used for the transcription experiments.

Transient transfection of the cells was carried out using Lipofectamine 2000 as the transfection agent and performed in quadruplicate. The transfection solutions were prepared as a 5 \times master mix since it was impractical to handle minute volumes of probes and transfection agent. Briefly, reconstituted solutions of plasmid DNA (500 ng for globally platinated probes and 50 ng for site-specifically platinated probes) in OptiMEM (62.5 μL) and lipofectamine 2000 (1.25 μL) in OptiMEM (62.5 μL) were prepared separately. After 5 min of incubation, the two solutions were combined and incubated for 30 min, during which time the liposome–DNA complexation took place. The liposomal mixture was diluted with 250 μL of antibiotics-free culture media (DMEM with 10% FBS) to yield 375 μL of 5 \times master mix transfection solution.

After transfection, synchronized XPF and XPF_{corr} cells were washed twice with antibiotics-free culture media (100 μL). Transfection solutions (75 μL) were transferred to each well, and the cells were incubated for 2 h. To remove the transfection solution, cells were washed twice with pre-warmed antibiotics-free culture media (100 μL) and returned to the incubator. This time point was designated as the start of the experiment ($t = 0$). For experiments involving the site-specifically platinated probes, cell media were collected at $t = 24 \text{ h}$. For experiments involving globally platinated probes, cell media were collected at 8 h intervals over a 48 h period, and the media were replaced with fresh pre-warmed antibiotics-free culture media. Cell media from untransfected cells were also collected as background control. The collected cell media were stored at 4 °C.

GLuc Reporter Gene Assays. GLuc expression levels were quantitated by using an assay that measures bioluminescence as a function of GLuc enzymatic activity in the presence of coelenterazine substrate. Collected cell media (10 μL) were transferred to 96-well white opaque assay plates. A luminescence plate reader (BioTek), fitted with reagent auto-injectors, was employed to assay GLuc activity of each individual well. Briefly, a GLuc assay solution (25 μL , 10 mM Tris·HCl pH 7.8, 1 mM EDTA, 600 mM NaCl, 10 μM coelenterazine) was injected into each well. The plate was allowed to shake for 5 s, after which a luminescence reading was taken. The readings were corrected against background readings taken from untransfected cells.

Acknowledgment. This work was supported by grant CA032134 from the National Cancer Institute. The authors thank Prof. Gan Wang (Wayne State University) for a donation of XPF-corrected XPF cells. W.H.A. acknowledges the National University of Singapore for an Overseas Postdoctoral Fellowship.

Supporting Information Available: Preparation and characterization of site-specifically platinated DNA plasmids. This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA101495V