

## Monofunctional Platinum–DNA Adducts Are Strong Inhibitors of Transcription and Substrates for Nucleotide Excision Repair in Live Mammalian Cells

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### Abstract

To overcome drug resistance and reduce the side effects of cisplatin, a widely used antineoplastic agent, major efforts have been made to develop next generation platinum-based anticancer drugs. Because cisplatin–DNA adducts block RNA polymerase II unless removed by transcription-coupled excision repair, compounds that react similarly but elude repair are desirable. The monofunctional platinum agent pyriplatin displays antitumor activity in mice, a cytotoxicity profile in cell cultures distinct from that of cisplatin, and a unique *in vitro* transcription inhibition mechanism. In this study, we incorporated pyriplatin globally or site specifically into luciferase reporter vectors to examine its transcription inhibition profiles in live mammalian cells. Monofunctional pyriplatin reacted with plasmid DNA as efficiently as bifunctional cisplatin and inhibited transcription as strongly as cisplatin in various mammalian cells. Using repair-defective nucleotide excision repair (NER)-, mismatch repair-, and single-strand break repair-deficient cells, we show that NER is mainly responsible for removal of pyriplatin–DNA adducts. These findings reveal that the mechanism by which pyriplatin generates its antitumor activity is very similar to that of cisplatin, despite the chemically different nature of their DNA adducts, further supporting a role for monofunctional platinum anticancer agents in human cancer therapy. This information also provides support for the validity of the proposed mechanism of action of cisplatin and provides a rational basis for the design of more potent platinum anticancer drug candidates using a monofunctional DNA-damaging strategy. *Cancer Res*; 72(3); 790–800. ©2011 AACR.

### Introduction

*cis*-Diamminedichloroplatinum(II) (cDDP, cisplatin) and its close analogues carboplatin and oxaliplatin are used to treat about half of all patients receiving chemotherapy for cancer (Fig. 1; ref. 1). Broader application of platinum-based anticancer drugs, however, is limited by intrinsic or acquired drug resistance and side effects including emetogenesis, nephrotoxicity, and neurotoxicity. Extensive efforts have been made to synthesize and test new platinum-based anticancer agents, with the promise that compounds with improved antitumor activity and fewer toxic side effects will be discovered.

The mechanism of action of platinum(II) anticancer agents has been extensively explored, and many details of the cellular response to these compounds are now understood. Cisplatin attacks nuclear DNA to form Pt–DNA cross-links. The major

adducts are intrastrand *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> cross-links including 1,2-d(GpG) cross-links, which efficiently block RNA polymerase II until removed by DNA damage repair pathways (2, 3). Besides the conventional bifunctional platinum-based anticancer drugs that contain 2 reactive sites for DNA binding, another class of compounds, monofunctional platinum anticancer agents with only one DNA reactive site, have been developed and tested. Monofunctional platinum(II) complexes such as chlorodiethylenetriamineplatinum(II) chloride, [PtCl(dien)]Cl and [PtCl(NH<sub>3</sub>)<sub>3</sub>]Cl do not inhibit DNA-dependent polymerases and are ineffective against cancer cells (4, 5). Another monofunctional compound, the aminophosphine-containing platinum(II) complex, *cis*-[PtCl(C<sub>6</sub>H<sub>11</sub>NH(CH<sub>2</sub>)<sub>2</sub>PPh<sub>2</sub>-*N,P*)(C<sub>6</sub>H<sub>11</sub>NH(CH<sub>2</sub>)<sub>2</sub>PPh<sub>2</sub>-*P*)], binds rapidly to DNA forming monodentate adducts at guanine residues. Although this compound has shown anticancer activity, it only slightly inhibits DNA synthesis and has little influence on DNA conformation (6). Some monofunctional platinum(II)–polyamide complexes, designed for recognition of specific DNA sequences, are capable of forming covalent bonds with DNA, but failed to evoke better cytotoxicity against cancer cells than cisplatin (7, 8). A series of cationic monofunctional platinum(II) complexes, *cis*-[Pt(NH<sub>3</sub>)<sub>2</sub>(N-donor)Cl]<sup>+</sup>, have been synthesized and analyzed, and some of the compounds showed moderate biological activity against *in vivo* murine tumor models (9). Like cisplatin, these complexes inhibit DNA polymerase despite their very different DNA-binding modality (10). The spectrum of activity of one of these compounds,

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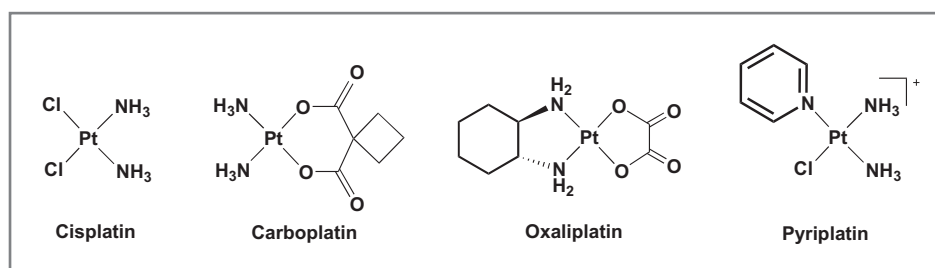
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Figure 1. Chemical structures of platinum(II) anticancer agents cisplatin, carboplatin, oxaliplatin, and pyriplatin.



*cis*-diammine(pyridine)chloroplatinum(II) [cDPCP or "pyriplatin" (Fig. 1)], against a panel of human cancer cell lines differs significantly from those of cisplatin or oxaliplatin, rendering pyriplatin a lead compound for generating new platinum anticancer drug candidates (11).

When attached to the N7 position of a guanine residue in duplex DNA, pyriplatin generates no significant structural distortion (12). An *in vitro* study revealed that pyriplatin is a substrate for nucleotide excision repair (NER), but it eludes this repair pathway much more readily than cisplatin. Studies of RNA polymerase II (pol II) activity on a DNA duplex containing a single *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct revealed a transcription inhibition mechanism distinct from that of bifunctional platinum compounds like cisplatin (13). An X-ray crystal structure analysis indicated that pyriplatin bound to the N-7 position of a guanosine residue can be accommodated in the pol II active site in which it forms a standard Watson-Crick base pair with cytosine of the growing RNA strand. Blockage of subsequent pol II translocation from the damaged site leads to inhibition of the pol II transcribing complex. In contrast, for the *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link, delivery of the damaged nucleosides to the active site is inhibited by a translocation barrier (14). Details of transcription inhibition by pyriplatin in live mammalian cells, especially in comparison to other bifunctional platinum-based anticancer drugs, and the repair pathways that are responsible for removal of pyriplatin-DNA adducts, remain to be elucidated, however.

To address these deficiencies, we have in this study investigated the transcription inhibition profiles of pyriplatin and compared them with those of cisplatin and oxaliplatin. We examined the ability of cisplatin, oxaliplatin, and pyriplatin to inhibit the transcription of a *Gaussia* luciferase reporter gene, utilizing globally platinated expression vectors in live mammalian cells. Different repair-deficient cell lines, including NER-, mismatch repair (MMR)-, and single-strand break repair (SSBR)-deficient cells, were used to reveal repair pathways that might be involved in removal of pyriplatin-DNA adducts. In addition, a site-specific pyriplatin-dG adduct was incorporated into the *Gaussia* luciferase expression vector. The transcription inhibition effects from this single pyriplatin-dG adduct in a 3,986-bp plasmid, as well as the mechanisms by which the repair-deficient cells process the site-specific lesion, were investigated. Our results shed light on the transcription inhibition effects and repair mechanisms of pyriplatin-DNA adducts. Moreover, they provide details about the mechanisms by which this monofunctional platinum compound generates its antitu-

mor activity and suggest how this activity can be improved in the design of novel anticancer drug candidates based on monofunctional platinum complexes.

## Materials and Methods

### Preparation of globally platinated transcription probes

For global platinated experiments, 125 μg/mL (45.4 nmol/L) of pGLuc, prepared as described in Supplementary Information, was treated with 0, 0.25, 0.51, 1.02, 2.04, and 4.07 μmol/L cisplatin; 0, 0.23, 0.45, 0.91, 1.81, and 3.63 μmol/L oxaliplatin; or 0, 0.42, 0.84, 1.68, 3.36, and 6.71 μmol/L pyriplatin in 25 mmol/L Na-HEPES, 10 mmol/L NaCl, pH 7.4 buffer for 16 hours at 37°C in the dark. A control plasmid without platinum was treated similarly. The reaction mixtures were then dialyzed against water and subsequently against TE buffer (10 mmol/L Tris-HCl, 2 mmol/L EDTA, pH 8.0) to remove unbound platinum. Quantification of Pt content for these globally platinated plasmids was obtained by flameless atomic absorption spectroscopy on a Perkin-Elmer AAnalyst 600 system. DNA concentrations were measured by UV-visible absorption spectroscopy at 260 nm on an HP 8453 UV-visible spectrometer. The number of platinum complexes bound per nucleotide,  $r_b$ , was computed from this information.

### Preparation of a pyriplatin modified insertion strand

A 16-mer oligonucleotide containing a site-specific *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct was prepared in the following manner. A 25.7 mmol/L aqueous solution of pyriplatin was activated by addition of 0.98 equivalent of AgNO<sub>3</sub> followed by agitation for 8 hours in the dark at room temperature. The suspension was centrifuged. To a 0.2 mmol/L solution of 5'-CCTCCTCG\*TCTCTTCC (Integrated DNA Technologies), where the asterisk denotes the base to be platinated, in 10 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, pH 6.3, was added 1.2 equivalent of activated pyriplatin. The reaction mixture was incubated overnight in the dark at 37°C. The reaction was stopped by freezing the solution. The pyriplatin-modified insertion strand was purified by ion exchange high-performance liquid chromatography [Agilent 1200 HPLC system, Dionex DNAPac PA-100, linear gradient, 0.34 to 0.45 mol/L NaCl in 25 mmol/L Tris-HCl (pH 7.4) over 11 minutes]. After purification, the platinated DNA solution was dialyzed against H<sub>2</sub>O and lyophilized. The platinated level was confirmed by UV-visible and atomic absorption spectroscopy, which yielded a Pt/DNA ratio of 1.03 ± 0.01. The insertion strand was further analyzed for nucleotide composition by enzyme digestion to confirm the

validity of the platination site following a previously published protocol (data not shown) (15).

#### Preparation of site-specifically platinated plasmids

Site-specifically platinated pGLuc8stemG plasmid containing a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct between the cytomegalovirus (CMV) promoter and the luciferase expression gene was prepared following the strategy published previously (16). The gapped plasmid was obtained with the use of Nt.BbvCI and Nt.BspQI nicking restriction enzymes (New England Biolabs), followed by annealing with excess 24-mer complementary strand to remove the nicked strand. A 300 µg quantity of pGLuc8stemG plasmid was digested with 15 U of Nt.BbvCI at 37°C for 1 hour. The reaction mixture was heated at 80°C for 20 minutes to deactivate the enzyme and then extracted with phenol/chloroform/isoamyl alcohol to remove the enzyme. The resulting aqueous phase was dialyzed against H<sub>2</sub>O overnight at 4°C. The plasmid was further digested with 15 U Nt.BspQI at 50°C for 1 hour, and the enzyme was heat deactivated and removed by a phenol/chloroform/isoamyl alcohol extraction. The nicked plasmid was mixed with 1000 equivalent of complementary DNA strand 5'-TTTTGGAAGAGACGAGGAGGTTTT in a buffer of 10 mmol/L Tris-HCl, 2 mmol/L MgCl<sub>2</sub>, 0.4 mol/L NaCl, pH 7.4, heated at 80°C for 5 minutes, and subsequently cooled at 4°C for 5 minutes for 10 cycles. The gapped plasmid was purified by isopycnic centrifugation using a CsCl gradient at 58,000 rpm, 20°C for 24 hours, and quantitated by UV-visible spectroscopy.

The 16-mer insertion strands, either containing a site-specific *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct or no platinum, were phosphorylated with T4 polynucleotide kinase at 37°C for 3 hours. The enzyme was removed by a phenol/chloroform/isoamyl alcohol extraction. The phosphorylated strands were ethanol precipitated and stored at -80°C at a concentration of 100 pmol/µL. A 120 µg quantity of the gapped plasmid was annealed with 100 equivalent of the insertion strand in a buffer of 10 mmol/L Tris-HCl, 2 mmol/L MgCl<sub>2</sub>, 0.4 mol/L NaCl, pH 7.4 from 90°C to 4°C at -1°C/min in a thermocycler, followed by a ligation with 240 U T4 DNA ligase at 16°C for 16 hours. The platinated plasmid was dialyzed against H<sub>2</sub>O at 4°C overnight and further purified by treatment with 30 U of BsmBI at 55°C for 1 hour. The closed circular form of plasmid was purified and concentrated by isopycnic centrifugation, followed by n-butanol extraction and ethanol precipitation. The plasmids were quantitated by a Quant-iT PicoGreen dsDNA kit (Invitrogen) and stored at -80°C in TE buffer.

To carry out a restriction analysis on ligated platinated or unplatinated plasmids, a 60 ng quantity of pGLuc8stemG plasmid was incubated with 2 U BsmBI at 55°C for 30 minutes. The plasmids were analyzed using 0.8% agarose gel electrophoresis containing 0.5 µg/mL ethidium bromide. The gels were documented with a BioRad Fluor-S MultiImager.

#### Cell lines and tissue culture

XPF (GM08437) cells were obtained from the Coriell Cell Depositories at Coriell Institute. XPFcorr cells were generously offered by Dr. Gan Wang at Wayne State University. U2OS cells were obtained from ATCC, and their derivatives U2OS-MOCK

and XPF-1128 cells were provided by Dr. Nora Graf in the Department of Chemistry at the Massachusetts Institute of Technology. PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> mouse embryonic fibroblasts (MEFS) were kindly provided by Prof. Paul Chang at the Massachusetts Institute of Technology. HEC59 and HEC59+Chr2 cells were obtained from Dr. Thomas Kunkel from NIH.

All cells were grown in a humidified incubator at 37°C under 5% CO<sub>2</sub>. XPF, U2OS-MOCK, and XPF-1128 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, and 1% penicillin/streptomycin. XPFcorr cells were maintained in DMEM with 10% FBS, 0.5 mg/mL G418 sulfate, and 1% penicillin/streptomycin. HEC59 cells were grown in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. HEC59+Chr2 cells were grown in DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.4 mg/mL G418 sulfate. PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> MEFS were maintained in DMEM medium with 10% FBS and 1% penicillin/streptomycin.

#### Transient transfection of cells and GLuc reporter transcription assays

Transfection of the platinated plasmids into mammalian cells was carried out as reported previously (17). The details are reported in the Supplementary Information section.

## Results

#### Transcription inhibition strategy

To investigate the extent to which pyriplatin-DNA adducts inhibit transcription and to gauge possible mechanisms for repairing its monofunctional adducts, transcription assays were carried out using platinated mammalian expression vectors in live mammalian cells of different origin (Supplementary Table S1). A *Gaussia* luciferase expression vector, pGLuc, which encodes a secretable form of the enzyme under control of a CMV promoter, was employed. Pyriplatin was incorporated into pGLuc either globally or site specifically between the CMV promoter and the luciferase gene. Platinated and unplatinated control plasmids were transfected into cells with cationic liposomes. Subsequently, the cell media containing the secreted luciferase were collected at various time intervals. An advantage of the secreted luciferase system is that a time-dependent cellular response to the platinated plasmids can be monitored without lysing the cells, as is necessary using other internal reporter enzyme systems (18, 19). The transcription inhibition activity of pyriplatin, and of cisplatin and oxaliplatin as controls, was determined by quantification of expressed luciferase using coelenterazine as substrate. NER-, MMR-, and SSB-deficient cells were employed both to monitor transcription inhibition activity of pyriplatin and to identify potential repair mechanisms of pyriplatin-DNA adducts in live cells.

#### Construction of globally platinated plasmids

pGLuc vectors were globally platinated with different platinum anticancer agents by allowing the plasmids to react with varying concentrations of the compounds in buffer. Platination levels were determined by atomic absorption and UV-visible

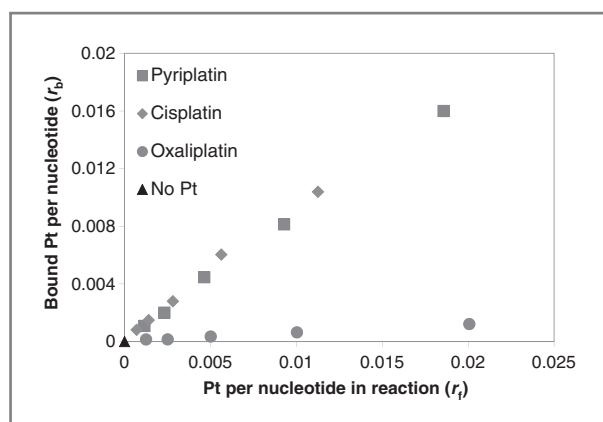


Figure 2. Plots of  $r_b$  versus  $r_f$  determined for pyriplatin, cisplatin, and oxaliplatin by pGLuc plasmid DNA.

spectroscopy (12). In Fig. 2, the amount of platinum bound per nucleotide ( $r_b$ ) in the reaction is plotted against the formal ratio of platinum to nucleotide ( $r_f$ ) for cisplatin, oxaliplatin, and pyriplatin. The slope of the  $r_b$  versus  $r_f$  plot for pyriplatin is identical to that of cisplatin, but much larger than that for oxaliplatin. In other words, pyriplatin reacts with DNA as efficiently as cisplatin, and both compounds react more efficiently than oxaliplatin.

### Construction of a plasmid containing a site-specific pyriplatin monofunctional adduct

A GLuc vector containing a site-specific *cis*- $\{Pt(NH_3)_2(py)\}^{2+}$ -dG adduct was constructed following the "gapping" strategy reported previously (16). pGLuc was modified to include 2 unique nicking restriction sites, Nt.BbvCI and Nt.BspQI, for incorporation of 16-mer insertion strands containing either a site-specific *cis*- $\{Pt(NH_3)_2(py)\}^{2+}$ -dG adduct or no platinum (Fig. 3). This new plasmid, pGLuc8temG, was then digested with the nicking restriction enzymes Nt.BbvCI and Nt.BspQI to obtain the desired gapped plasmid. The 16-mer insertion strands, containing either a site-specific pyriplatin adduct or no platinum, were annealed and ligated into the gapped plasmids to form the final constructs. The site-specificly platinated and unplatinated control plasmids were designated as pGLuc8temG+IS-PtPy and

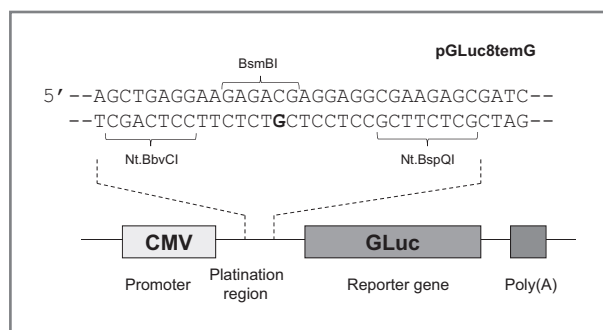


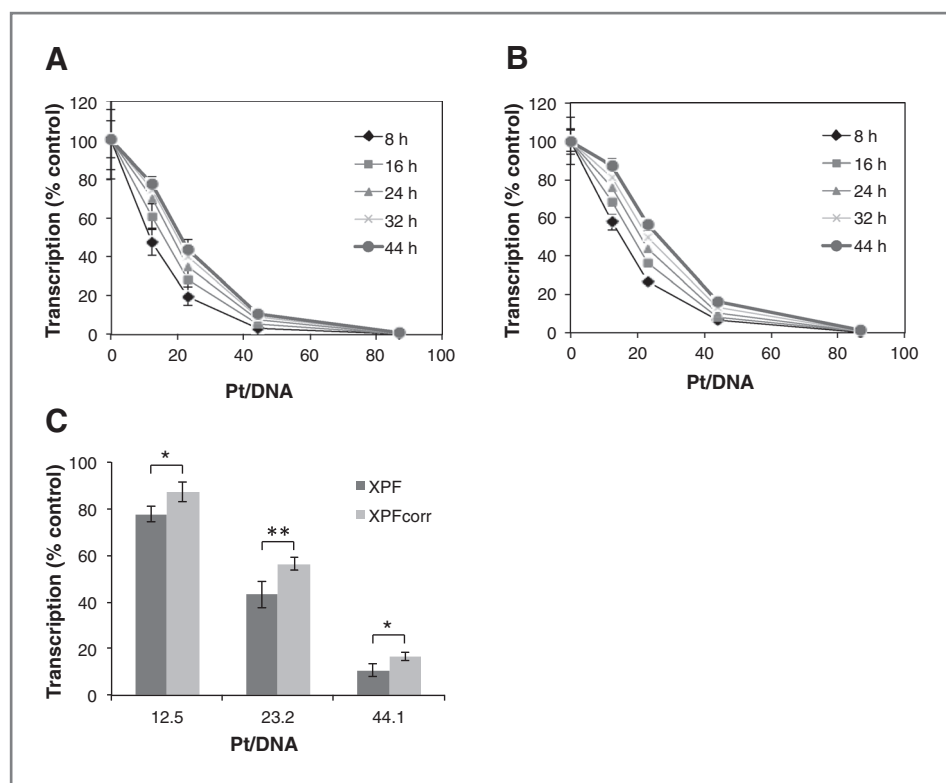
Figure 3. DNA sequence for building a site-specifically platinated *Gaussia* luciferase reporter containing a pyriplatin-dG adduct; the platinated site is highlighted in bold.

pGLuc8temG+IS, respectively. Restriction analysis with BsmBI was carried out to confirm the presence of the monofunctional pyriplatin-DNA adduct, and the result is presented in Supplementary Fig. S1. The site-specific pyriplatin adduct in the BsmBI restriction site efficiently inhibits the restriction digestion.

### Transcription inhibition profiles of pyriplatin in NER-deficient cells

Transcription inhibition profiles were obtained for globally and site-specifically platinated probes in NER-deficient cells. Plasmids were transfected into XPF cells, which lack the *XPF* gene, and into XPFcorr cells, in which the XPF function was restored by introduction of the cDNA for XPF (20). pGLuc globally platinated with pyriplatin was examined first, and expression levels were read after 8, 16, 24, 32, and 44 hours of incubation. GLuc expression levels normalized to that of unplatinated plasmid were plotted as a function of Pt/DNA ratios at various time points (Fig. 4), or as a function of time at specific Pt/DNA ratios (Supplementary Fig. S2). After 44 hours, the transcription levels were substantially restored in both cell lines, indicating repair of pyriplatin-DNA adducts. There was stronger transcription inhibition by pyriplatin in XPF cells. For example, at a Pt/DNA ratio of 23.2, the transcription level recovered from 19.8% at 8 hours to 43.4% at 44 hours in XPF cells, whereas in XPFcorr cells, transcription recovered from 27.0% at 8 hours to 56.3% at 44 hours. In addition,  $D_0$  values, where  $D_0$  is the number of Pt adducts per plasmid required to reduce transcription levels to 37% of control (21), were obtained to quantitate transcription inhibition differences between the 2 cell lines (Table 1). The increase in  $D_0$  values at different time points indicates restoration of transcription, and the higher  $D_0$  values in XPFcorr cells suggest a role for NER in repairing pyriplatin-DNA damage.

Transcription profiles of site-specifically platinated pGLuc in XPF and XPFcorr cells were also determined. The transcription levels from a plasmid carrying a single, site-specific pyriplatin-DNA adduct were 37%, 49%, and 54% in XPF cells, and 80%, 115%, and 123% in XPFcorr cells at 24, 48, and 72 hours, respectively. We also studied site-specific pGLuc plasmids containing a *cis*- $\{Pt(NH_3)_2\}^{2+}$  intrastrand 1,2-d(GpG) cross-link, the major type of cisplatin-DNA adduct, as a control. Transcription levels in the presence of the *cis*- $\{Pt(NH_3)_2\}^{2+}$  1,2-d(GpG) cross-link were 53%, 59%, and 62% in XPF cells and 102%, 128%, and 132% in XPFcorr cells at 24, 48, and 72 hours, respectively (Fig. 5). Transcription levels more than 100% after 48 hours in XPFcorr cells for both cisplatin and pyriplatin suggest, perhaps, the stimulation of transcription from newly repaired plasmids. Restoration of XPF function significantly decreased the transcription inhibition induced by a site-specific pyriplatin-dG adduct, further confirming that NER can remove pyriplatin adducts on DNA. The results from a *cis*- $\{Pt(NH_3)_2\}^{2+}$  1,2-d(GpG) cross-linked plasmid are in agreement with our previously published data (17), reinforcing the pivotal role of NER in the repair of cisplatin-DNA cross-links. It is noteworthy that a *cis*- $\{Pt(NH_3)_2(py)\}^{2+}$ -dG monofunctional adduct inhibits transcription better than a *cis*- $\{Pt(NH_3)_2\}^{2+}$  1,2-d(GpG) cross-link in XPF cells.



**Figure 4.** Transcription profiles of globally platinated probes with pyriplatin in XPF (A) and XPFcorr (B) cells at 8 hours (◆), 16 hours (■), 24 hours (▲), 32 hours (×), and 44 hours (●). C, transcription inhibition by pyriplatin at average loadings of 12.5, 23.2, and 44.1 platinum atoms per plasmid at 44 hours in XPF and XPFcorr cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

Osteosarcoma cancer cells (U2OS) with downregulated XPF gene expression (22) were also examined. Transcription profiles of plasmids globally platinated with pyriplatin or oxaliplatin in XPF-normal (U2OS-MOCK) and XPF-knockdown (XPF-1128) cells are shown in Fig. 6 and Supplementary Figs. S3, S4, and S5. For oxaliplatin, recovery of transcription occurred in both cell lines in a similar manner over a 44-hour time period (Supplementary Fig. S4), and there was stronger transcription inhibition in XPF-1128 cells (Supplementary Fig. S5). For pyriplatin, there was little evidence of transcription recovery in U2OS-MOCK cells, and almost none in XPF-1128 cells (Supplementary Fig. S3). Stronger transcription inhibition was also observed in XPF-1128 cells (Fig. 6). Calculated  $D_0$

values for both oxaliplatin and pyriplatin are tabulated in Supplementary Table S2. At most time points, pyriplatin has only slightly smaller  $D_0$  values than oxaliplatin in U2OS-MOCK and XPF-1128 cells.

#### Transcription inhibition profiles of pyriplatin in MMR-deficient cells

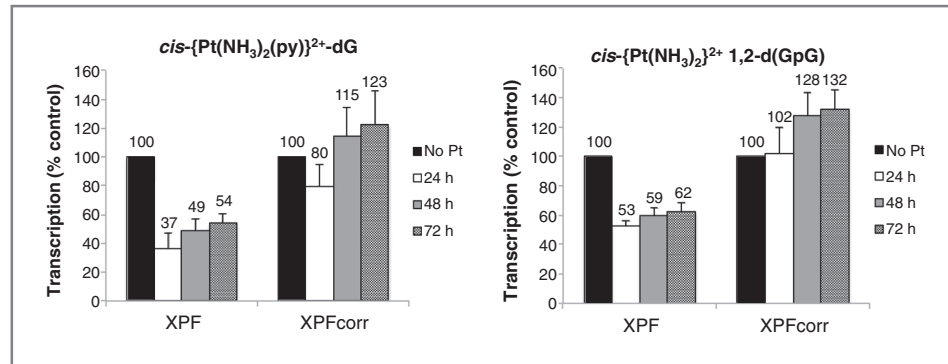
In addition to cells deficient in NER, MMR-deficient (HEC59) and MMR-proficient (HEC59+Chr2) cells were examined in transient transfection assays with globally platinated plasmids to investigate the potential role of MMR in removing Pt–DNA adducts, including those from cisplatin, oxaliplatin, and pyriplatin. For cisplatin, there was slightly more transcription inhibition in MMR-proficient compared with MMR-deficient cells over 44 hours (Supplementary Fig. S6). Transcription levels increased over 44 hours in both HEC59 and HEC59+Chr2 cells. At an adduct level of 19.4 Pt/DNA, transcription increased from 19.2% to 35.2% of control in HEC59 cells and from 10.1% to 31.6% in HEC59+Chr2 cells (Supplementary Fig. S6). There was identical transcription inhibition of oxaliplatin in MMR-deficient and MMR-proficient cells, and little recovery over time in both cell lines (Supplementary Fig. S7). As for pyriplatin-modified transcription probes, there were identical transcription profiles in both MMR-deficient and MMR-proficient cells over 44 hours, and slightly greater transcription inhibition occurred in HEC59+Chr2 cells (Fig. 7).  $D_0$  values for cisplatin, oxaliplatin, and pyriplatin in both cell lines are shown in Supplementary Table S3. Pyriplatin and cisplatin have identical  $D_0$  values in HEC59 and HEC59+Chr2 cells, and the values are greater than those of oxaliplatin. There was a small

**Table 1.**  $D_0$  values<sup>a</sup> of globally platinated probes with pyriplatin assayed at different time intervals after transfection for XPF and XPFcorr cells

Time after transfection (h)	XPF (Pt/plasmid)	XPFcorr (Pt/plasmid)
8	14.12 ± 2.82	17.45 ± 1.35
16	17.30 ± 2.61	19.93 ± 0.86
24	19.60 ± 1.99	21.86 ± 1.21
32	21.22 ± 1.91	23.85 ± 1.23
44	22.34 ± 2.05	26.16 ± 1.28

<sup>a</sup> $D_0$  value is defined as the number of Pt lesions per plasmid required to reduce transcription levels to 37% of the control.

**Figure 5.** Transcription profiles of site-specifically platinated probes containing a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct or a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) intrastrand cross-link in XPF and XPFcorr cells.



increase in D<sub>0</sub> from 8 to 44 hours in both cell lines for cisplatin, oxaliplatin, and pyriplatin. D<sub>0</sub> values were slightly smaller in MMR-proficient cells, especially for cisplatin and pyriplatin.

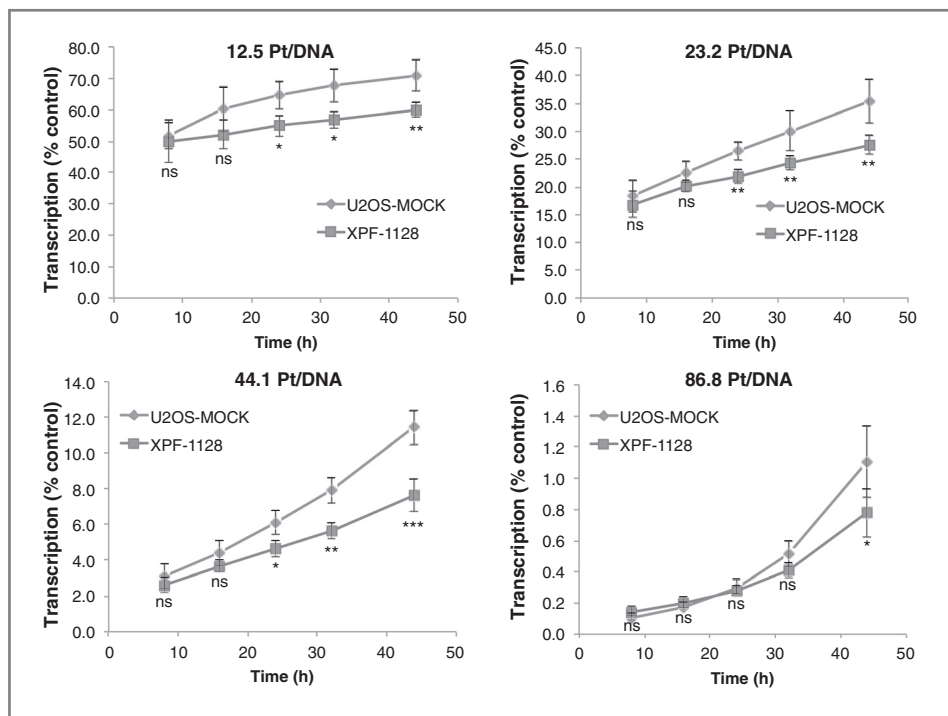
Transcription assays with site-specifically platinated plasmids showed identical results to those obtained for globally platinated transcription probes. Site-specific pGLuc plasmids containing a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link or a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG monofunctional adduct were examined. Transcription levels were determined after 24, 48, and 72 hours (Supplementary Fig. S8). The transcription inhibition effects of the *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct were the same as those of a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link in both HEC59 and HEC59+Chr2 cells. There was a small increase in transcription inhibition by pyriplatin in HEC59+Chr2 cells. The transcription levels in the presence of a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link were 66%, 76%, and 90% in HEC59 cells and 65%, 76%, and 89% in HEC59+Chr2 cells at 24, 48, and 72 hours, respectively (Supplementary Fig. S8).

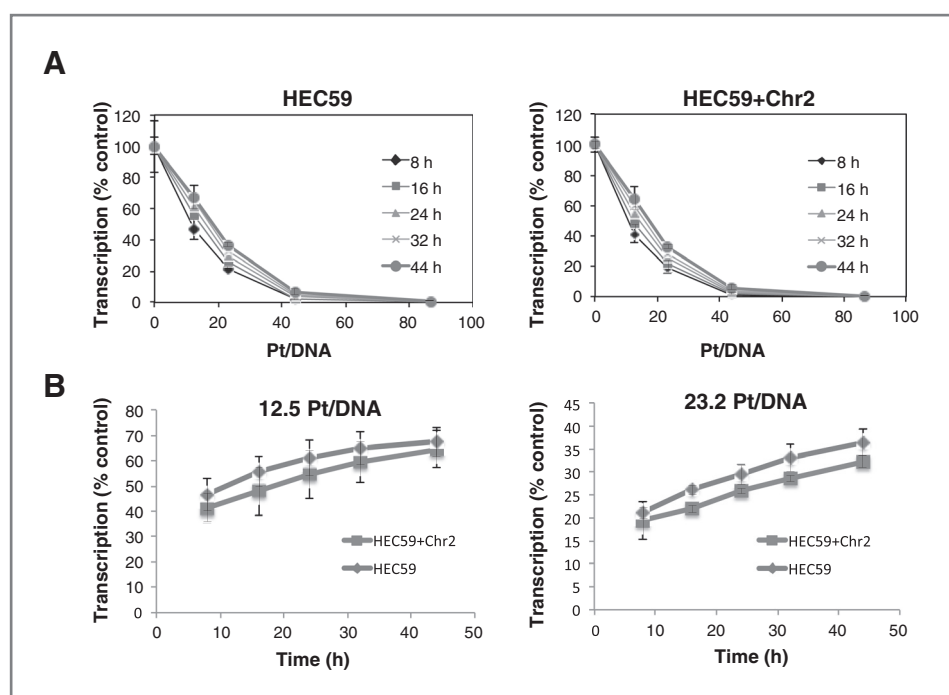
**Transcription inhibition profiles of pyriplatin in PARP-1 knockdown cells**

PARP-1 knockdown MEFS were studied to reveal the transcription inhibition profiles of cisplatin, oxaliplatin, and pyriplatin. A small recovery in transcription levels was observed for all 3 platinum compounds tested, especially in PARP-1<sup>+/+</sup> cells (Supplementary Figs. S9 and S10). Pyriplatin had D<sub>0</sub> values identical to those of cisplatin and oxaliplatin in PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells (Supplementary Table S4), further indicating that pyriplatin inhibits transcription as strongly as cisplatin and oxaliplatin. A slight increase of D<sub>0</sub> values occurred from 8 and 44 hours in both cell lines for cisplatin, oxaliplatin, and pyriplatin, and the D<sub>0</sub> values were slightly smaller in PARP-1<sup>-/-</sup> cells.

The transcription profiles of site-specifically platinated pGLuc in PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells were also determined. Transcription levels in the presence of a site-specific *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct were 46%, 56%, and 76% in

**Figure 6.** Transcription inhibition by pyriplatin at average loadings of 12.5 (top, left), 23.2 (top, right), 44.1 (bottom, left), and 86.8 (bottom, right) platinum atoms per plasmid in U2OS-MOCK and XPF-1128 cells; ns, not significant; \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.





**Figure 7.** Transcription inhibition by pyriplatin in MMR-deficient (HEC59) and MMR-proficient (HEC59+Chr2) cells. **A**, transcription profiles of globally platinated probes as a function of Pt adducts/DNA at 8 hours (◆), 16 hours (■), 24 hours (▲), 32 hours (×), and 44 hours (●). **B**, transcription inhibition of pyriplatin at 12.5 and 23.2 Pt adducts/DNA as a function of time.

PARP-1<sup>+/+</sup> cells and 61%, 72%, and 77% in PARP-1<sup>-/-</sup> cells at 24, 48, and 72 hours, respectively. The transcription levels in the presence of a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link were 52%, 60%, and 67% in PARP-1<sup>+/+</sup> cells and 61%, 77%, and 84% in PARP-1<sup>-/-</sup> at 24, 48, and 72 hours, respectively (Supplementary Fig. S11). A *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct inhibited transcription as well as a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link in both PARP-1<sup>+/+</sup> and PARP-1<sup>-/-</sup> cells.

## Discussion

Transcription inhibition is one of the major consequences of platinum–DNA damage, and there is a correlation between transcription inhibition by platinum compounds and their efficacy as anticancer agents (23, 24). Numerous reports have presented the transcription inhibition of bifunctional platinum antitumor compounds through reconstituted systems or studies in cell extracts or culture. Different mechanisms of transcription inhibition have been proposed, including hijacking of transcription factors, physical blocking of RNA polymerases, and disruption of chromatin structure (24). Knowledge of transcription inhibition at monofunctional platinum–DNA adducts in the cell is, however, very limited. Monofunctional adducts at guanine residues formed by *cis*-[PtCl(NH<sub>3</sub>)<sub>2</sub>(N7-ACV)] [ACV, acyclovir or 9-(2-hydroxyethoxymethyl)guanine], an active antiviral and antitumor compound, terminate DNA and RNA synthesis *in vitro* (25, 26). Other monofunctional platinum(II) compounds, such as [PtCl(dien)]Cl and [Pt(NH<sub>3</sub>)<sub>3</sub>Cl]Cl, or monofunctional adducts of cisplatin, were unable to inhibit RNA (27–29) or DNA (5) polymerase activity *in vitro*. Pyriplatin effectively blocked RNA synthesis *in vitro*, and a detailed mechanism of how this monofunctional plat-

inum antineoplastic compound can inhibit pol II has been put forth (13). This study significantly extends our knowledge of transcription inhibition by monofunctional platinum anticancer agents to live mammalian cells and provides important information about which repair pathways remove monofunctional Pt–DNA adducts.

## Transcription inhibition of pyriplatin in NER-deficient cells

NER is considered the major pathway for removing cisplatin–DNA 1,2-d(GpG) intrastrand cross-links (3, 30, 31). There are 2 subpathways in NER, namely, transcription-coupled NER (TC-NER) and global genome NER. TC-NER-deficient cells are hypersensitive to cisplatin, indicating its critical role in the platinum–DNA damage response (30). The transcription inhibition profile of a reporter gene containing global cisplatin cross-links, or a site-specific cisplatin cross-link, is dramatically different in XPF and XPFcorr cells (17). Here, we studied the transcription inhibition profiles of oxaliplatin and pyriplatin in NER-deficient cells including XPF cells. Transcription of a globally pyriplatin-damaged reporter gene recovers slightly over 44 hours, and greater transcription inhibition occurs in XPF cells, indicating a role of NER in the repair of pyriplatin–DNA damage. Compared with the transcription inhibition of cisplatin in XPF and XPFcorr cells that we reported previously (17), however, pyriplatin does not evoke significant transcription recovery with time when globally platinated plasmids were used, indicating that pyriplatin–dG adducts are more difficult for the cellular machinery to recognize and repair. We also incorporated pyriplatin site specifically into the pGLuc plasmid between the CMV promoter and the GLuc

expression gene to evaluate whether a pyriplatin–DNA adduct can act as a roadblock to the transcribing pol II complex in live cells. Robust transcription inhibition by a single pyriplatin–dG adduct was observed in XPF cells. Restoration of the XPF function significantly restored the transcription levels of luciferase from a plasmid site-specifically modified with pyriplatin. Identical results were obtained from plasmids globally platinated with pyriplatin in XPF-knockdown cells, strongly supporting our conclusion that NER plays a key role in the repair of pyriplatin–DNA damage. Moreover, pyriplatin inhibited transcription as effectively as oxaliplatin in U2OS cells, despite their NER-deficient status.

#### Transcription inhibition of platinum compounds in MMR-deficient cells

MMR corrects single base mismatches and looped intermediates generated from DNA polymerase slippage during replication and recombination, as well as some forms of DNA damage by endogenous or exogenous toxicants (32). DNA mismatches are recognized by hMutS $\alpha$ , a heterodimer of hMSH2 and hMSH6, and hMutS $\beta$ , a heterodimer of hMSH2 and hMSH3. The binding of MMR proteins to different types of platinum–DNA adducts has been studied *in vitro*. hMSH2 binds with some specificity to DNA globally damaged by cisplatin, and hMutS $\alpha$  recognizes cisplatin 1,2-d(GpG) but not 1,3-d(GpTpG) cross-links (33–35). Cell-based studies revealed that MMR-deficiency is correlated with cisplatin resistance (36, 37). This line of evidence, together with other findings, implies that, in addition to the role of MMR in platinum damage repair, MMR proteins bind to cisplatin–DNA damage and initiate a signal transduction pathway leading to cell-cycle arrest and apoptosis (38).

We examined endometrial HEC59 (hMSH2 deficient) and HEC59+Chr2 (hMSH2 proficient) adenocarcinoma cells to investigate the transcription inhibition of pyriplatin as well as the potential role of MMR in the repair or processing cisplatin, oxaliplatin, and pyriplatin lesions. Transcription assays with globally platinated plasmids indicated the inhibitory effects of cisplatin and pyriplatin to be greater in hMSH2-proficient cells. When  $D_0$  values are considered, pyriplatin is as active as cisplatin in inhibiting transcription of a reporter gene, and oxaliplatin is the most active compound in these cells. Platinum–DNA adducts from cisplatin and pyriplatin are slightly better transcription inhibitors in hMSH2-proficient cells, and no significant difference was observed between hMSH2-deficient and hMSH2-proficient cells when oxaliplatin plasmids were employed (Supplementary Table S3). These results further show that MMR may play a role, albeit a minor one, in mediating the cytotoxicity of cisplatin and pyriplatin. The results are in agreement with a previous model in which MMR proteins bind to cisplatin–DNA cross-links but not those from oxaliplatin (39). When site-specifically platinated plasmids were used, there were identical transcription inhibition effects for a cisplatin 1,2-d(GpG) cross-link in hMSH2-deficient and hMSH2-proficient cells. A *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct, which is as active as a cisplatin 1,2-d(GpG) cross-link in blocking RNA synthesis,

had a moderately stronger transcription inhibition effect in hMSH2-proficient cells. Taken together, these results suggest that MMR, instead of being the major repair pathway for removing pyriplatin–DNA adducts, may play a role in initiating apoptosis signaling pathways in response to pyriplatin–DNA damage.

#### Transcription inhibition of platinum compounds in PARP-1-knockdown cells

PARP-1 is one of the most abundant nuclear proteins in eukaryotes. The cellular functions of PARP-1 include modulation of chromatin structure and transcription, as well as DNA repair housekeeping (40). When genomic DNA is mildly damaged, PARP-1 is fully activated to recruit repair machinery and signal downstream effectors (41, 42). PARP-1 mainly detects single-strand breaks (SSB), obligatory intermediates in base excision repair (BER) and NER. PARP-1 thus participates actively in BER, and there is accumulating evidence for a role in NER (43). The affinity of PARP-1 for cisplatin-damaged DNA has been established by photocross-linking studies, and *in vitro* binding assays further revealed the binding of PARP-1 to DNA damaged by cisplatin, oxaliplatin, and pyriplatin (44–47). The transcription inhibition effects of pyriplatin in PARP-1 knockdown cells and the question of whether or not PARP-1 has a significant function in the repair of pyriplatin–DNA adducts in live cells have never been explored until now.

To investigate these possibilities, we used PARP-1-knockdown MEFS to reveal the transcription inhibition profile of pyriplatin and the possible role of PARP-1 in repairing pyriplatin–DNA adducts. Our results from globally platinated plasmids show that pyriplatin has identical  $D_0$  values compared with cisplatin and oxaliplatin; thus pyriplatin, once it binds to DNA, inhibits transcription as efficiently as these U.S. Food and Drug Administration–approved platinum drugs. Cisplatin and oxaliplatin display greater transcription inhibition in PARP-1<sup>-/-</sup> cells at both high and low numbers of platinum adducts on the plasmids, implying a role for PARP-1 in repair of cisplatin- or oxaliplatin-damaged DNA (Supplementary Fig. S9). When plasmids globally platinated with pyriplatin were examined, transcription inhibition levels were not notably different for PARP-1 normal versus knockdown cells, especially at 12.5 and 23.2 Pt/DNA (Supplementary Fig. S10), suggesting that SSBR may not be a determinative repair pathway in response to pyriplatin damage. We also used site-specifically platinated plasmids in this reporter assay. Transcription inhibition effects were slightly lower in the PARP-1 knockdown cells for plasmids containing a site-specific *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link or a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>-dG adduct (Supplementary Fig. S11). Nevertheless, the transcription inhibition effects of the monofunctional platinum dG adduct are as strong as those of the bifunctional platinum 1,2-d(GpG) cross-link. One explanation for the difference between the results from globally and site-specifically platinated plasmids is that PARP-1 is not fully activated by a single platinum present on the plasmid. The protein may still bind to the adduct, however, possibly shielding the damage from repair. Such an outcome would result in



greater transcription inhibition in PARP-1 normal cells. The results from globally and site-specifically platinated plasmids suggest that SSBR is not the major repair pathway to remove pyriplatin–DNA adducts.

### Implications for the mechanism of action of cisplatin and suggestions for improvement of monofunctional platinum anticancer agents

The mechanism of cisplatin action is a multistep process including cell entry, activation, DNA binding, and subsequent cellular responses (2, 3). Cisplatin is transported across the plasma membrane by both passive diffusion and carrier-mediated active transport. Once cisplatin enters cells, the relatively low chloride ion concentration of the cytosol favors the formation of activated aquated species. This active form of cisplatin subsequently attacks nucleophilic centers such as DNA bases, which are mainly responsible for the cytotoxicity of the drug. Cisplatin can also bind other molecules such as glutathione or metallothionein, processes that deactivate the compound. Formation of the platinum–DNA cross-links induces a complex series of cellular responses, including transcription inhibition, cell-cycle arrest, and apoptosis. Remarkably, the monofunctional compound pyriplatin seems to have a mechanism of action very similar if not identical to that of cisplatin. This commonality of mechanism, despite dramatically different structures for the Pt–DNA adducts, provides support for the current consensus mechanism of the platinum anticancer drugs (1).

Previous reports reveal that pyriplatin is an excellent substrate for organic cation transporters (OCT; ref. 12) but that the potency of pyriplatin is lower than that of cisplatin against ovarian, breast, and a variety of other types of cancer cells (11). From the present results, we conclude that pyriplatin binds to DNA in cancer cells as efficiently as cisplatin. Pyriplatin, once bound to DNA, inhibits transcription as strongly as cisplatin, and pyriplatin–DNA adducts are more difficult to remove by the cellular repair machinery. NER is mainly responsible for removal of pyriplatin–DNA adducts.

Because pyriplatin and cisplatin share similar DNA binding, transcription inhibition, and repair pathways, we propose 3 possible reasons to account for the lower cytotoxicity of pyriplatin compared with cisplatin. First, the cell may not accumulate pyriplatin as well as cisplatin. Although taken up by OCTs, pyriplatin may not pass as readily into the cytoplasm and nucleus of the cell. Second, pyriplatin may be deactivated more readily prior to DNA binding. Third, transcription inhibition by pyriplatin may not correlate with its cytotoxicity, which we believe to be unlikely in mammalian systems. This analysis, combined with previous results on the structures of site-specifically modified DNA, including a transcription complex with pol II, has guided the rational design of significantly more potent monofunctional platinum anticancer agents (12, 13). In particular, as will be described elsewhere, monofunctional cationic platinum complexes bearing more extended aromatic fused ring heterocyclic ligands than pyridine have recently been obtained that significantly exceed the level of cisplatin cytotoxicity (G.Y. Park and S.J. Lippard, unpublished data). Such compounds are currently being evaluated in pre-

clinical studies for possible development as next generation platinum anticancer drugs.

### Summary and Conclusion

In these studies, we established transcription inhibition profiles for pyriplatin–DNA adducts in NER-, MMR- and SSBR-deficient cells as well as in the corresponding repair-proficient cells. *Gaussia* luciferase reporters were used to measure the effects of pyriplatin modification of DNA, and plasmid-based reporter assays allowed us to examine transcription inhibition by pyriplatin and the DNA repair capacity in live cells. Pyriplatin reacts with plasmid DNA as efficiently as cisplatin and significantly more so than oxaliplatin. It inhibits transcription in live cells to the same extent as cisplatin when globally platinated plasmids were used. A single, site-specific *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>(py)}<sup>2+</sup>–dG adduct within the 3,986-bp plasmid dramatically inhibits transcription, at least as strongly as a *cis*-{Pt(NH<sub>3</sub>)<sub>2</sub>}<sup>2+</sup> 1,2-d(GpG) cross-link. NER, rather than MMR or SSBR, plays the most important role in removing pyriplatin–DNA adducts. We provide the first evidence that a monofunctional platinum anticancer agent can block RNA synthesis as efficiently as traditional bifunctional platinum anticancer drugs in live mammalian cells, and our data suggest that the repair mechanism of the monofunctional platinum compounds might be similar to that of bifunctional platinum compounds. Our findings support a role for monofunctional platinum anticancer agents in cancer chemotherapy. Because pyriplatin can react with DNA to a level similar to that of cisplatin and, when bound to DNA, inhibit transcription as effectively as cisplatin, monofunctional platinum compounds with improved cellular uptake and reduced ability to bind cellular targets not associated with cytotoxicity may prove to be a promising route to novel platinum-based therapies.

### Disclosure of Potential Conflicts of Interest

S.J. Lippard is a cofounder and serves as head of the Scientific Advisory Board of Blend Therapeutics, a newly formed biopharmaceutical company which is developing nanoparticle drug combinations that may include platinum complexes. The other authors disclosed no potential conflicts of interest.

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