Cisplatin-Induced Nucleosome and RNA Polymerase II Modifications Mediate Cellular Response to the Drug

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Cisplatin-Induced Nucleosome and RNA Polymerase II Modifications Mediate
Cellular Response to the Drug

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Abstract

Chapter 1. Cellular Processing of Platinum Anti-Cancer Drugs - Identifying
Pathways for Chemogenotherapeutic Drug Design

Cisplatin, carboplatin, and oxaliplatin are widely used in cancer
chemotherapy. Platinum-DNA adducts, formed following uptake of the drug
into the nucleus of cells, activate several cellular processes that mediate the
cytotoxicity of these platinum drugs. This review focuses on recently discovered
cellular pathways that are activated in response to cisplatin, including those
involved in regulating drug uptake, the signaling of DNA damage, cell cycle
checkpoints and arrest, DNA repair, and cell death. Such knowledge of the
cellular processing of cisplatin adducts with DNA provides valuable clues for the
rational design of more efficient platinum-based drugs as well as the
development of new therapeutic strategies.

Chapter 2. Nucleotide Excision Repair from a Site-Specifically Platinum-
Modified Nucleosome

Nucleotide excision repair is a major cellular defense mechanism against
the toxic effects of the anticancer drug cisplatin and other platinum based
chemotherapeutic agents. In this study, mononucleosomes were prepared
containing either a d(GpG) or a d(GpTpG) intrastrand cross-link. Comparison of
the extent of repair by mammalian cell extracts of free and nucleosomal DNA
containing the same platinum-DNA adduct reveals that the nucleosome
significantly inhibits nucleotide excision repair. The effects of post-translational
modification of histones on excision of platinum damage from nucleosomes were
investigated by comparing native and recombinant nucleosomes containing the
same intrastrand d(GpTpG) cross-link. Excision from native nucleosomal DNA is
\sim 2\text{-}fold higher than the level observed with recombinant material. The in vitro
system established in this study will facilitate the investigation of platinum-DNA
damage by DNA repair processes and help elucidate the role of specific post-
translational modification in NER of platinum-DNA adducts at the physiologically relevant nucleosome level.

Chapter 3. Nucleotide Excision Repair of Site-Specifically Platinum-Modified Tetrasomes

The nucleosome, the basic structural unit of chromatin, is composed of a histone (H3/H4)2 tetramer flanked by two H2A/H2B dimers, around which is wrapped 146 base pairs (bp) of DNA. The (H3/H4)2 tetramer plays a central role in the structural integrity and positioning of the nucleosome core particle. Site-specifically platinated tetrasomes were prepared to investigate the modulating effects of histone tetramers on excision repair of cisplatin-intrastrand cross-links. In addition, [Pt(DACH)]2+-modified tetrasome was prepared to investigate the effect of spectator ligands on excision repair of platinated tetrasomes. The NER results reveal that the (H3/H4)2 tetramer is sufficient to block excision of both cisplatin-DNA and [Pt(DACH)]2+-DNA adduct in vitro. The efficiency of excision of cisplatin-modified tetrasomal DNA is about half (53%) that of the [Pt(DACH)]2+-modified tetrasome.

Chapter 4. Cisplatin Adducts Change the Rotational Positioning of DNA on the Nucleosome

Understanding the interaction of cisplatin-DNA adducts with nucleosomes and the effect of cisplatin-DNA adduct formation on nucleosome structure is important for improving the cytotoxicity of this drug. Here we use hydroxyl radical and DNAse I footprinting analysis to characterize site-specifically cisplatin-modified nucleosomes. Preexisting cisplatin-DNA adducts change the rotational setting of DNA with respect to the histone core particle surface during nucleosome formation.

Chapter 5. Cisplatin-Induced Post-Translational Modification of Histones H3 and H4

The anticancer drug cisplatin kills cells by damaging DNA and inducing apoptosis. Understanding the detailed mechanisms by which cancer cells respond to cisplatin has the potential to improve substantially platinum-based therapy. Post-translational modification of histones alters chromatin structure, facilitating the binding of nuclear factors that mediate DNA repair, transcription and other processes. In the present study, we have investigated the effects of cisplatin treatment on histone post-translational modification in cancer cells. We discovered that specific phosphorylation of histone H3 at Ser 10, mediated by the p38 MAPK pathway, is induced in response to cisplatin treatment. In addition,
hyperacetylation of histone H4 is caused by the drug treatment. These findings reveal a link between cisplatin administration and chromosomal structural alterations, providing mechanistic information about how cells respond to platinum-induced stress.

Chapter 6. The Modulating Effect of DNA Methylation on the Cellular Processing of Cisplatin-Damaged DNA

Epigenomic regulation of genes is essential for proper cellular function. One major way of conveying epigenetic information in mammalian cells is DNA methylation, with 5-methylcytosine within the CpG dinucleotide being the major component. DNA methylation has profound effects on the mammalian genome. Cancer cells frequently exhibit altered genomic methylation patterns, mostly exhibiting global hypomethylation with region-specific hypermethylated sequences that occur within the promoter region of certain genes in these cells. Here we investigate the role of DNA methylation on the processing of cisplatin-modified DNA, specifically, the role of DNA methylation in the formation of DNA-platinum adducts as well as on protein-DNA interactions and on the repair of platinum damage. The rates and extent of platination of DNA probes containing 5-methylcytosine residues adjacent to the platinum binding site were compared to similar properties of non-methylated DNA. The effects of DNA methylation on the interaction between a protein and platinated DNA were investigated by studying HMGB1 domA binding with methylated and non-methylated probes. Finally, the effect of DNA-methylation on the NER of cisplatin-damaged DNA was examined with 195mer substrates containing site-specific cisplatin-DNA cross-links with flanking or base-paired 5-methylcytosine residues.

Chapter 7. Transcription-Coupled and DNA Damage-Dependent Ubiquitination of RNA Polymerase II In Vitro

Transcription-coupled repair (TCR) is essential for the rapid, preferential removal of DNA damage in active genes. The large subunit of RNA polymerase (Pol) II is ubiquitinated in cells following UV-irradiation or cisplatin treatment, which induces DNA damage preferentially repaired by TCR. Several human mutations, such as Cockayne syndrome CSA and CSB, are defective in TCR and incapable of Pol II ubiquitination upon DNA damage. Here we demonstrate, for the first time, a correlation between ubiquitination of RNA Pol II and arrest of transcription in vitro. Ubiquitination of Pol II is significantly induced by alpha-amanitin, an amatoxin which blocks Pol II elongation and causes its degradation in cells. Pol II undergoes similar ubiquitination on DNA containing cisplatin
adducts that arrest transcription. Stimulation of ubiquitination requires the addition of template DNA, and it does not occur in the presence of an antibody to the general transcription factor TFIIB, indicating the transcription dependence of the reaction. We propose that components of the reaction recognize elongating polymerase-DNA complexes arrested by alpha-amanitin or cisplatin lesions, triggering ubiquitination.

In the second part, site-specifically platinated-DNA probes for in vitro transcription studies were synthesized by both primer-extension and enzymatic ligation methods. In vitro study revealed that the presence of cisplatin-DNA adducts on the template strand significantly block the RNA polymerase.

Chapter 8. Synthesis and Characterization of Platinated Dumbbell DNAs for In Vitro Transcription and Repair Studies

Site-specifically platinated double-stranded DNA probes were prepared as valuable templates for in vitro studies of cellular processing of cisplatin, indicating the formation of protein-DNA complexes, transcription, and repair. However, the blunt-ends of double-stranded probes permit non-specific transcription, protein-binding, and exonuclease degradation. Here we report the synthesis of site-specifically platinated dumbbell DNA probes from a pair of hairpins with 3' overhangs and three or four central fragments using enzymatic ligation. Formation of the modified dumbbell DNA probes were confirmed by exonuclease and restriction enzyme digestion assays. The dumbbell DNA probes were then used as templates for in vitro nucleotide excision repair and transcription studies.

Appendix A1. The Interaction of Human SWI/SNF with Platinated Nucleosomes and Its Effect on NER

In eukaryotic cells, DNA is packaged into chromatin. Two major classes of factors have been identified to alter DNA accessibility in chromatin: histone modified complexes and ATP-dependent remodeling complexes. SWI/SNF, one of best characterized members of the latter class, has been linked to nucleotide excision repair stimulation. The stimulatory effect of SWI/SNF on NER depends upon the nature of the DNA lesion. SWI/SNF stimulates the excision of AAF-adducts and the (6-4) photoproduct from nucleosomal DNA, whereas it has a negligible effect on the repair of TT dimers. These differential effects of SWI/SNF on the repair of UV photoproducts and AAF adducts promoted our interest in investigating the effect of SWI/SNF complexes on repair of cisplatin damage within the nucleosome. In this section, the effect of SWI/SNF on the repair of cisplatin-DNA adducts in a mononucleosome will be addressed. A moderate
stimulatory effect of SWI/SNF on the repair from cisplatin-DNA adducts was observed.

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Title: Arthur Amos Noyes Professor of Chemistry
For my mother, my farther, and my sister,
who taught me to love, to care,
and many other things.

And for Francis Harry Compton Crick (1916-2004),
who revealed such a perfect structure,
and inspired millions of people.
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Chapter 1  Cellular Processing of Platinum Anti-Cancer Drugs - Identifying Pathways for Chemogenotherapeutic Drug Design*

* Submitted as an invited paper in *Nature Reviews Drug Discovery*. 
Introduction

Cisplatin and carboplatin have been widely for many years to treat several kinds of cancer, including as testicular, ovarian, head and neck, and non-small cell lung. Over this period, thousands of platinum analogues have been synthesized and screened for anti-cancer activity. Among them, the only one that has been approved by the FDA is oxaliplatin, for treatment of colorectal cancer. Several interesting platinum compounds, including those with a trans geometry at the platinum center, platinum(IV) molecules with tethered substituents, and multinuclear species, have been reported to be as active as cisplatin in one or another assay, as has been reviewed elsewhere.

Although many cellular components interact with cisplatin, DNA is the primary biological target of the drug. The platinum atom of cisplatin forms covalent bonds to the N7 positions of purine bases to form primarily 1, 2- or 1, 3-intrastrand cross-links and a fewer number of interstrand cross-links. These linkages to DNA occur at the positions where the chloride, CBDCA, or oxalate ligands reside in the original platinum drug. Structural studies of cisplatin and oxaliplatin DNA adducts have been reviewed elsewhere.

The abbreviations used are: cisplatin, cis-diaminedichloroplatinum(II); trans-DDP, trans-diaminedichloroplatinum(II); carboplatin, cis-diammine(cyclobutane-1,1-dicarboxylato)-platinum(II); CBDCA, cyclobutane-1,1-dicarboxylate; ER, estrogen receptor; ATM, ataxia-telangiectasia mutated; tsHMG, testes-specific HMG box protein; ATR, ataxia-telangiectasia and Rad3-related; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; NER, nucleotide excision repair; TCR, transcription-coupled repair; GGR, global genomic repair; MMR, mismatch repair; XIAP, X-linked inhibitor of apoptosis; MDM2, murine double minute-2; MEF, mouse embryo fibroblasts; PARP, poly(ADP-ribose)polymerase; SCC, serum squamous cell; Cdc25C, cell division cycle 25C; PCNA, proliferating cell nuclear antigen; GADD, growth arrest and DNA damage; ES, embryonic stem.
Cisplatin-DNA adducts cause a wide variety of cellular responses (Figure 1.1).\textsuperscript{8} Understanding the mechanisms of how cells process cisplatin provide important insights for designing more efficient platinum-based drugs. For example, the discovery that treatment of ER(+) cells with estradiol sensitizes them to cisplatin inspired the design and synthesis of a series of active estrogen-tethered \textit{Pt(IV)} compounds.\textsuperscript{9} Information of this kind facilitates the introduction of new families of molecules in which the fundamental genotoxicity of a platinum-DNA adduct can be made selective for cancer cells by targeting the compound selectively to tumors or by interrupting a pathway used by the cancer cell to survive the chemical assault. The current trend among cancer biologists to downplay the importance of chemotherapy in favor of pathway-directed modalities is, in our opinion, failing to take advantage of a time-proven weapon against the disease. Combining the best of chemotherapeutic action with specific targets in cellular pathways offers a powerful new approach to cancer treatment that perhaps may impede the many clever ways that human cells have in becoming drug-resistant.

This purpose of the present review is to highlight recent discoveries in those pathways involved in regulating platinum drug transport, DNA damage signaling, cell cycle checkpoints and arrest, DNA repair and mechanisms of cell death. From the understanding of these cellular pathways it has become possible to form a biological basis for the chemical synthesis of new platinum drugs for cancer treatment, a process that we term chemogenotherapy.
Mechanisms of Cisplatin Transport into Cells

The mechanisms of cisplatin cellular uptake and efflux are still not fully understood. Early studies suggested that cisplatin enters the cell mainly by passive diffusion, since its uptake proceeded linearly with time up to 60 min and could not be saturated up to a concentration of 1.0 mM. In addition, cisplatin absorption was not inhibited by structural analogues and there did not appear to be an optimum pH for its entry into the cell.\textsuperscript{10-12}

More recently, a growing body of evidence reveals a link between Ctr1p, a high-affinity copper transporter, and cisplatin uptake (Figure 1.2). Mutation or deletion of the \textit{CTR1} gene results in increased cisplatin resistance and reduction of platinum levels in both yeast and mouse cells.\textsuperscript{13} Moreover, \textit{CTR1}-deficient cells exhibit impaired accumulation of cisplatin analogues carboplatin, oxaliplatin, and ZD0473[cis-amminedichloro(2-methylpyridine)platinum(II)].\textsuperscript{14,15}

Another direct link between the cellular management of copper and platinum concentrations comes from studies of cisplatin efflux. Copper-transporting P-type adenosine triphosphate (ATP7B), which plays an important role in regulating copper levels in the cell, is associated with cisplatin resistance in vitro.\textsuperscript{16} Moreover, ATP7B levels correlate with cisplatin resistance in a variety of cancers. For example, in the case of primary oral serum squamous cell (SCC) carcinoma, patients with \textit{ATP7B}-positive tumors have reduced overall survival following cisplatin treatment than those who are \textit{ATP7B}-negative.\textsuperscript{20} The
expression level of the *ATP7B* gene was significantly increased in patients with undifferentiated ovarian carcinoma and gastric carcinoma, which is less sensitive to cisplatin treatment.\(^{17-19}\) Although *ATP7A* is expressed in a considerable fraction of many common tumor types, its role is less clear; however, enrichment of human ovarian cancer cells that express *ATP7A* is associated with poor response to platinum-based therapy.\(^{21}\) In addition, MRP2 (cMOAT), an exporter protein, also plays a role in cisplatin resistance, presumably by promoting drug efflux. Overexpression of *MRP2* is associated with increased cisplatin resistance.\(^{22-24}\)

The observation that both cisplatin uptake and efflux are seemingly linked to the copper metabolic pathway leads to the hypothesis that copper and cisplatin might interfere with their mutual transport.\(^{25}\) In fact, both copper and cisplatin have the ability to reduce the uptake of the other and can trigger the degradation and delocalization of Ctr1p.\(^{13}\) Moreover, copper and cisplatin also exhibit bidirectional cross-resistance.\(^{26,27}\) Expression of *ATP7B* in human carcinoma cells modulates sensitivity to both cisplatin and copper by causing more rapid efflux of the two agents.\(^{16}\)

These observations offer a potential new mechanism for targeting platinum to tumor cells by taking advantage of an emerging receptor for delivering the drug into the cancer cell. Any compound having a substituent that can direct the platinum complex to a tumor or organ tissue population in a specific manner is of potential interest for chemogenotherapy. Included in this
category would be molecules that target the tumor vasculature\textsuperscript{28-31} or deliver the molecule specifically to cells having a receptor for the appended moiety.\textsuperscript{9,32}

**Cisplatin-DNA Damage Recognition Proteins - A Postulated Key Role for HMGB1**

Cisplatin modifications distort the structure of the DNA duplex, bending it significantly toward the major groove and exposing a wide, shallow minor groove surface to which several classes of proteins bind. Included are high mobility group box proteins, repair proteins, transcription factors, and other proteins such as histone H1, which preferentially recognize 1,2-intrastrand cross-linked platinum-DNA adducts, as summarized elsewhere.\textsuperscript{1,33-35} Here we focus on recent progress in understanding the functions of the HMG box family of proteins and their putative role in facilitating the anticancer activity of cisplatin.

The abundant HMG box protein, HMGB1, also named Amphoterin, has been connected to several DNA-dependent pathways (Figure 1.3). HMGB1 stimulates RAG1/2 cleavage in V(D)J recombination and promotes binding of sequence-specific transcription factors.\textsuperscript{36-38} In addition, HMGB1 physically interacts with MutS\textalpha{} and plays a role in mismatch repair.\textsuperscript{39} Moreover, HMGB1 enhances p53 DNA binding activity and directly interacts with p53 in vitro.\textsuperscript{40} Both the C-terminal and A box domains of HMGB1 regulate p53-mediated transcription activation and its downstream effects.\textsuperscript{41} HMGB1 affinity for
cisplatin-damaged DNA is significantly enhanced by p53. Interaction between p53 and HMGB1 after platination may provide a molecular link between DNA damage and p53-mediated DNA repair. In vitro nucleotide excision repair studies indicate that HMG box proteins such as HMGB1 and tsHMG shield the Pt-1,2-d(GpG) cross-link from repair proteins by stably binding to this DNA adduct. Until recently, the postulated in vivo relationship between HMG box protein levels and cisplatin toxicity has been somewhat controversial. Several studies report that HMG box protein levels correlate with cisplatin sensitivity. For example, overexpression of HMGB1 induced by addition of the steroid hormone estradiol sensitizes breast and ovarian cancer cells to cisplatin treatment. Deletion of yeast Ixr1, a gene that encodes a transcriptional repressor containing two HMG boxes, results in 2-6-fold increased resistance to cisplatin, depending on the strain investigated. On the other hand, cancer cell lines that have developed resistance to cisplatin often have elevated levels of HMGB1. In addition, no significant difference in sensitivity to cisplatin was observed between and Hmgb1 knockout and wild type mouse embryonic fibroblast cell lines. Moreover, S. pombe mutant deficient in the HMGB protein Cmb1 is more sensitive to cisplatin treatment. These discrepancies most likely reflect the importance of cell type and history in determining the ability HMGB1 and related proteins to mediate cisplatin cytotoxicity. Recent work from our laboratory using RNAi clearly demonstrate the strong correlation with HMGB1 levels and the sensitivity of cells to cisplatin.
HMGB1 interacts with the nucleosome at the entry and exit points of DNA and facilitates binding of the remodeling complex ACF to nucleosomal DNA, thereby accelerating the ability of ACF to induce structural changes in the nucleosome. Of greater interest is the recent discovery that the dynamic behavior of HMGB1 is completely altered in apoptotic cells, where it binds irreversibly to chromatin, most likely due to the change of the nature of chromatin. In addition, HMGB1 plays an important role as a cytokine in the inflammatory response. In monocytes activated by inflammatory stimuli, HMGB1 relocalizes from the nucleus to the cytoplasm, and eventually to secretory organelles. HMGB1 thus appears to act as a signal of tissue damage, inducing mesoangioblast migration and proliferation.

The activity of HMGB1/2 is modulated by post-translational modifications. Histone acetyltransferase CBP acetylates HMGB1/2 proteins in vitro. The acetylated HMGB1/2 full-length protein is 3-fold more effective in inducing ligase-mediated circularization of a 111-bp DNA fragment than unmodified full length protein. In addition, acetylation of HMGB1 plays an important role in regulating relocalization of HMGB1 from nucleus to cytoplasm, and subsequent secretion when monocytes receive an appropriate second signal. The export of HMGB1 from necrotic cells does not appear to require such post-translational modification. If viable cancer cells surrounding the necrotic core of a solid tumor took up HMGB1, it could potentiate the ability of
cisplatin to kill such cells and account for the specificity of the drug for cancer cells.

The structure-specific recognition protein, SSRP1, contains a single HMG domain that was initially isolated from expression screening of a human B-cell cDNA library for proteins that bind to cisplatin-modified DNA. Human SSRP1 and Spt16 form a complex named FACT, which facilitates RNA polymerase II transcription elongation through a nucleosome block. The isolated HMG domain of SSRP1 and FACT, but not SSRP1 alone, can bind to the major 1,2-d(GpG) intrastrand cisplatin adduct, which suggests that Spt16 primes SSRP1 for cisplatin-damaged DNA recognition by unveiling its HMG domain. Also, SSRP1 functions as a co-activator of the transcriptional activator p63.

Yeast Nhp6, an abundant HMG box protein, is a component of yeast FACT complex in *S. cerevisiae*. Nhp6 is required for proper regulated expression of a number of genes that are transcribed by RNA polymerase II or III. Either Nhp6 alone or the yFACT complex were able to bind nucleosomes and significantly reorganize their structure. In addition, Nhp6Ap binds to cisplatin-modified intrastrand cross-links on duplex DNA with a 40-fold greater affinity than to unmodified DNA with the same sequence. Nhp6A/Bp appears to function directly or indirectly in yeast to enhance cellular resistance to cisplatin.
Cell Cycle Checkpoints and Arrest

Cell cycle checkpoints monitor the proper order of events in the cell. When DNA is damaged, the cell cycle is arrested to provide time for repair. Failures to do so leads to the acquisition and accumulation of genetic alterations, ultimately causing tumorigenesis.71

The G1/S checkpoint ensures that damaged DNA is not replicated. Following DNA damage, ATM (ataxia-telangiectasia mutated) cells may control the fate of a G1/S checkpoint through direct phosphorylation of p53 or through phosphorylation of c-Abl, which in turn upregulates p21 through p73 activation.72,73 ATM-dependent phosphorylation of p53 contributes to p53 stabilization by reducing its interaction with MDM2 (murine double minute-2).74,75 In addition, p21, a downstream target of p53 and a universal inhibitor of cyclin-dependent kinases, plays a major role in mediating p53-dependent cell cycle G1 arrest. p21 inhibits G1 cyclin/cdk5 and blocks cell cycle progression.76,77 Exogenous expression of p21 exerts cell growth inhibition and enhances sensitivity to cisplatin in hepatoma cells.78

Cisplatin does not cause G1 arrest in all cases, however. In fact, the drug fails to induce a G1 arrest response in synchronized wild-type MEF cells, even though it can activate p53 and cause an S phase arrest.79

The G2/M checkpoint allows for repair of DNA that was damaged in late S or G2 phase of the cell cycle prior to mitosis to prevent damaged DNA from
being segregated into daughter cells. It has been proposed that such G2 arrest is essential to the process of engaging cell death following cisplatin treatment.\textsuperscript{80,81} Upon the occurrence of DNA damage, ATM and its related ATR (ataxia-telangiectasia and Rad3-related) kinase activate checkpoint kinases Chk1 and 2 through phosphorylation, which in turn phosphorylate Cdc25C. The phosphorylated Cdc25C promotes its binding to 14-3-3 adaptor proteins and thereby is separated from Cdc2 by translocation of Cdc25c to the cytoplasm. As a result, Cdc2 phosphorylation is elevated and causes cells to arrest in G\textsubscript{2}.\textsuperscript{82-88}

In addition, p21 plays a regulatory role in maintaining cell cycle arrest at G2 by blocking the interaction of Cdc25C with PCNA.\textsuperscript{89-91} More recently, SHP-2 extra-cellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinases (p38 MAPK) have also been implicated in the G\textsubscript{2}/M cell cycle checkpoint.\textsuperscript{92-95} Therefore, it appears that multiple pathways contribute to the regulation of the G\textsubscript{2}/M checkpoint following genotoxic stress.

\textbf{p53 and p73 Pathways}

p53 is an important tumor suppressor protein which is mutated in greater than 50% of all human tumors.\textsuperscript{96} The p53 protein plays a crucial role in many cellular processes; in particular, it inhibits cell proliferation by inducing either cell cycle arrest or apoptosis in response to cellular stress. p53 functions as a transcription factor and upregulates a number of genes involved in these
processes (Figure 1.4). In the absence of cellular stress, p53 is maintained at low steady-state levels and exerts very little effect on the cell. Upon cellular stress, p53 is overexpressed and undergoes post-translational modification.

p53 has also been connected with DNA repair, specifically nucleotide excision repair (NER) and base excision repair (BER). One of the activities of p53 involves its transcriptional activation of the Gadd45 gene. GADD45 interacts with PCNA and stimulates nucleotide excision repair activity when overexpressed. p53 is also involved in repair through direct interactions with critical components of the NER machinery. XPC, an important damage recognition protein in NER, is induced after DNA damage in a p53-dependent manner. XPC also can help initiated cisplatin damage-mediated cell cycle regulation. In addition, p53 binds to three of the subunits of the TFIIH transcription/repair complex, XPB, XPD, and the p62 subunit, suggesting an additional role in NER. Moreover, p53 physically interacts with RPA. Such interaction is disrupted following UV or cisplatin damage in p53-wild type cells, but not in p53-mutant repair-deficient cells. These results suggest that release of RPA and p53 plays a role in facilitating NER. The repair defect in p53-mutant ovarian carcinoma cells IGROV-1/Pt may be attributed to a reduced removal/recycling of PCNA at repair sites.

Sensitivity to cisplatin correlates positively with the presence of wild-type p53 function in a National Cancer Institute (NCI) panel of 60 human tumor cell lines. In addition, tumor cell lines that lack functional p53 are more resistant to
cisplatin than cells that contain functional p53, but can be sensitized when reconstituted with wild-type p53. Other studies, however, show no or even negative correlation between p53 status and response to cisplatin. For example, p53 mutation promotes increased sensitivity to cisplatin, with loss of G1/S checkpoint control and decreased cisplatin-DNA adduct repair. Furthermore, one study demonstrated that, in SaOS-2 osteosarcoma cells, p53 function was associated with increased cisplatin sensitivity under high serum growth conditions but with decreased sensitivity under low serum conditions. Previous work from our own laboratory using murine testicular teratocarcinoma cells also revealed that, although rapid apoptosis is induced in p53-wild-type but not in p53-/- teratocarcinoma cells upon cisplatin treatment, the absence of p53 does not alter cellular sensitivity to cisplatin as measured in clonogenic assays. The relationship between p53 status and cisplatin cytotoxicity depends upon several factors, including tumor cell type, activation of specific signaling pathways, and the presence of other genetic alterations.

Besides p53, there are two other members of the p53 family, p63 and p73, which are homologous to p53 in terms of overall domain structure and conformation. Both p63 and p73 are expressed as multiple isoforms, truncated at the N- or C-terminus through alternative splicing and use of several promoters. p73 can cooperate with DNA damaging agents or p53 to induce some p53 target genes and activate their promoters. Signaling pathways for p53 and p73 in inducing cell cycle arrest and apoptosis are similar but also have
important differences. p73 differentially regulates some p53 target genes, either several fold lower than p53, such as p21, or several fold higher than p53, such as 14-3-3σ. DNA damage can stabilize p73 and enhance p73-mediated apoptosis in a c-Abl dependent manner. p73 upregulates MDM2 expression, but unlike p53, it is not targeted by MDM2 for ubiquitin-dependent degradation. Instead, MDM2 downregulates p73 transactivator function by disrupting its interaction with p300/CBP, a component of the eukaryotic transcription complex.

Proteins involved in p53 related pathways modulate p53 activity and cisplatin cytotoxicity. Aurora kinase A is a key regulatory component of the p53 pathway and phosphorylates p53 at Ser315, leading to its ubiquitination by Mdm2 and proteolysis. Aurora kinase A is overexpressed in many human cancers, which leads to increased degradation of p53, and down-regulation of checkpoint-response pathways. Silencing of aurora kinase A results in less phosphorylation of p53 at Ser315, greater stability of p53 and cell-cycle arrest at G2-M, and ultimately increased sensitivity to cisplatin treatment.

MDM2 interacts with the N-terminus of p53 and inhibits p53 transcriptional activity. In addition, MDM2 acts as the E3 ligase for p53 and thus promotes p53 ubiquitination and degradation. Post-translational modifications of p53 and MDM2, including phosphorylation, acetylation and sumoylation, modulate their interactions and thus influence p53 stability and activity. In addition, STAT-1 interacts directly with p53 acts as a co-activator for p53 and a negative regulator of Mdm2 expression.
Cyclin G is another transcriptional target gene of tumor suppressor p53. Cyclin G may play a central role in the p53-MDM2 autoregulated module. Inhibition of cyclin G by small interfering RNA (siRNA) causes the accumulation of p53 levels in response to DNA damage.\textsuperscript{126} This cyclin G-mediated p53 regulation is dependent upon the status of ATM protein, which activates p53 in response to DNA damage.\textsuperscript{127}

The human BRCA1 gene contains one RING finger domain and two BRCT domains. BRCA1 interacts directly or indirectly with numerous proteins involved in transcriptional regulation, cell cycle/checkpoint control, protein ubiquitination, chromatin remodeling, and DNA repair. BRCA1 functions as a molecular determinant of response to a range of cytotoxic chemotherapeutic agents.\textsuperscript{128} BRCA1 is a substrate of ATM kinase in vitro and in vivo, and is regulated by an ATM-dependent mechanism as a part of the cellular response to DNA damage.\textsuperscript{129} The p53 and BRCA1 tumor suppressors are involved in repair processes and may cooperate to transactivate certain genes, including p21\textsuperscript{WAF/CIP1} and GADD45. BRCA1 also enhances p53 binding to the DDB2 promoter in vivo. Antisense of BRCA1 abrogates upregulation of DDB2 after cisplatin exposure. DNA repair activity is significantly restored by introduction of BRCA1 into wild-type as compared to DDB2-deficient cells, suggesting that BRCA1 plays a role in DNA damage repair-mediated cisplatin resistance.\textsuperscript{130,131}

These studies demonstrate that many cellular functions controlled directly or indirectly by p53 respond to cisplatin treatment. Since p53 is not essential for
cisplatin-mediated cell-killing, however, it is unlikely that any one of them is critical to the selectivity and curative properties of the drug for certain cancers such as testicular.

**MAPK and Other Related Pathways**

*Akt Pathway*

Akt, protein kinase B (PKB), is a member of the family of phosphatidylinositol 3-OH-kinase-regulated serine/threonine kinases. Akt promotes cell survival and down-regulates apoptotic pathways through phosphorylation and modulation of several downstream target proteins. Therefore, Akt protects cells from apoptotic death induced by a variety of stimuli, including cisplatin.

Akt phosphorylates X-linked inhibitor of apoptosis (XIAP) and thus stabilizes XIAP by inhibiting both its auto-ubiquitination and cisplatin-induced ubiquitination activities. Increased levels of XIAP are associated with decreased cisplatin-stimulated caspase 3 activity and apoptosis. Inhibition of XIAP and/or Akt expression/function may be an effective means of overcoming chemoresistance in ovarian cancer cells expressing either endogenous or reconstituted wild-type p53. Akt also phosphorylates the MDM2 protein promoting its translocation to the nucleus and destabilization of p53, therefore impairing the cellular stress response and increasing the survival of tumor cells.
Akt also phosphorylates and inactivates several other apoptotic pathway proteins. For example, Akt phosphorylates BAD, promoting its association with 14-3-3 proteins in the cytosol and inactivating its proapoptotic function.\textsuperscript{134} Moreover, Akt phosphorylates and inactivates apoptosis signal-regulating kinase-1 and mixed lineage kinase 3 (MLK3), the mitogen-activated protein kinase kinase kinases (MAPKKK), leading to cell survival.\textsuperscript{138,139} Constitutively active Akt might be due to down-regulation of FADD-protein or the upregulation of the caspase 8 inhibitor c-FLIP to increase cisplatin resistance.\textsuperscript{140} Expression of adenovirus E1a gene is able to block selectively Akt activation mediated by cisplatin and increases sensitivity to cisplatin.\textsuperscript{140}

In addition, Akt also phosphorylates IκB kinase, subsequently activating nuclear factor of κB (NFκB). NFκB plays an important role in controlling cell survival.\textsuperscript{133} Increase of NFκB activity correlates with decreased apoptosis, whereas inhibition of NFκB increases the efficacy of cisplatin both in vitro and in vivo.\textsuperscript{133,141}

\textit{c-Abl and Bcr-Abl}

c-Abl is a member of the Src family of non-receptor tyrosine kinases, which contain Src- homologue regions 2 and 3 (SH2 and SH3) at N-terminus and a large C-terminus region that contains nuclear localization motifs and nuclear export sequences. In addition, c-Abl also carries a DNA binding motif and an actin-binding domain at its extreme C-terminus. Nuclear c-Abl tyrosine kinase
activity can be stimulated by cisplatin and other DNA damaging agents and acts to transmit DNA damage signals from the nucleus to the cytoplasm. The activation of c-Abl tyrosine kinase might be a downstream event in ATM-dependent apoptosis. ATM may directly phosphorylate c-Abl at Ser465 or indirectly phosphorylate c-Abl through DNA-PK or other unidentified protein kinases. c-Abl tyrosine kinase plays a role in the activation of apoptosis induced by cisplatin. Overexpression of c-Abl tyrosine kinase in Saos-2 cells and NIH3T3 cells can activate apoptosis, whereas c-Abl deficient mouse fibroblasts and chicken DT-40 cells are resistant to cisplatin-induced apoptosis. Re-introduction of a functional c-Abl in these Abl-deficient cells restore their sensitivity to the drug. In addition, knocking down c-Abl expression by RNA interference confers resistance to cisplatin-induced apoptosis.

Retinoblastoma tumor suppressor protein (RB) plays a negative role in DNA damage-induced activation of c-Abl kinase. RB binds to the tyrosine kinase domain of c-Abl, thereby blocking c-Abl tyrosine kinase activity and preventing c-Abl from being activated by DNA damage signals. Phosphorylation of RB at G1/S disrupts the RB/c-Abl interaction, leading to the release of c-Abl in the nucleus of S phase cells.

The transcription factors p73 and p53 are possible downstream effectors of c-Abl. Cisplatin can induce the accumulation of p73 protein in wild type and p53-deficient, but not in c-Abl-deficient, primary mouse embryo fibroblasts. Furthermore, c-Abl phosphorylates p73 both in vitro and in vivo, and the SH3
domain of c-Abl can directly bind to and phosphorylate the p73 protein.\textsuperscript{148,149} c-Abl is not essential to the induction and activation of p53 by DNA damage, although c-Abl can stabilize and activate p53 in transient co-transfection experiments.\textsuperscript{150}

c-Abl is also an upstream effector of MEKK-1 and the stress-activated kinases, c-Jun N-terminal kinases (JNK) and p38 MAPK.\textsuperscript{151-153} The treatment of wild-type fibroblasts, but not Abl-/- cells, with cisplatin is associated with an increased level of tyrosine phosphorylation of MEKK-1.\textsuperscript{154} In addition, cells deficient in c-Abl fail to activate p38 MAP kinase and JNK after treatment with cisplatin, but not after exposure to UV and methyl methanesulfonate. Reconstitution of c-Abl in the Abl-/- cells restores the activation of p38 MAPK and JNK.\textsuperscript{155} These findings indicate that c-Abl dependent activation of p38 MAP and JNK protein kinases is specific to cisplatin. p38 MAPK and JNK are differentially regulated in response to different classes of DNA-damaging agents.

Another interesting feature of c-Abl is its ability to associate with chromatin. c-Abl contains three HMG-like boxes that preferentially bind to A/T duplexes and distorted DNA structures, such as four-way junctions. Whether c-Abl could recognize and interact with cisplatin-DNA adducts like HMGB1 is a very interesting question, the answer to which might provide more insight into c-Abl activation by the drug.\textsuperscript{156,157}

Different subcellular localizations might contribute to the opposing effects of c-Abl and Bcr-Abl kinase on apoptosis.\textsuperscript{158} Cytoplasmic Bcr-Abl can inhibit
apoptosis through the activation of Akt. Mutagenesis assays demonstrated that cells expressing a deletion mutant of BCR/ABL that lacked the SH2 and SH3 domains of ABL (BCR/ABLΔΔ) were sensitive to cisplatin. Moreover, Bcr-Abl tyrosine kinase can be converted into a potent inducer of apoptosis by allowing it to function in the nucleus, which is consistent with the role of the nuclear c-Abl tyrosine kinase in DNA damage-induced cell death.

**MAPK/JNK/ERK Pathways**

The MAPK family of proteins play important roles in signal transduction processes through specific phosphorylation cascades in response to a variety of extracellular stimuli. There are three major mammalian MAPK subfamilies, ERK, JNK (also called stress-activated protein kinase), and p38 MAPK. The ERK pathway is highly induced in response to growth factors and cytokines, and it is also activated by some conditions of stress, particularly oxidative stress. In contrast, JNK and p38 are highly activated in response to a variety of stress signals including ionizing and short wavelength ultraviolet irradiation (UVC), tumor necrosis factor, and hyperosmotic stress, and they are only weakly activated by growth factors. Cisplatin triggers the activation of the ERK, JNK and p38 MAPK cascades in tumor cells or transformed cell lines (Figure 1.5), as discussed in following section.
p38 MAPK Pathway

A growing body of evidence suggests that activation of the p38 MAPK pathway plays an important role in the cellular response to cisplatin.\textsuperscript{155,161-163} Cisplatin, but not trans-diamminedichloroplatinum(II) (trans-DDP) or PtCl\textsubscript{4}, induces sustained activation of p38 MAPK.\textsuperscript{161} Cisplatin activates p38 MAPK for 8-12 hr in sensitive cells and for 1-3 hr in resistant cells.\textsuperscript{164} This difference in the kinetics of activation could account for their differential cytotoxicity. Moreover, lack of p38 MAPK activation/function induces a resistant phenotype in human cells.\textsuperscript{161,164} MAP kinases may also play a role in modifying the chromatin environment of target genes.\textsuperscript{165} This kinase regulates immediate-early (IE) gene expression and other cellular responses through phosphorylation of various substrates, including transcription factors, chromatin protein constituents, and downstream Ser/Thr effector kinases.\textsuperscript{166,167} Furthermore, p38 MAP kinase and downstream kinase MSK-1 phosphorylate histone H3 Ser in vitro.\textsuperscript{167} The p38 MAPK pathway is also involved in cisplatin-induced phosphorylation of histone H3 at Ser 10.\textsuperscript{168}

ERK Pathway

Cisplatin treatment results in dose- and time-dependent activation of ERK1/2.\textsuperscript{169-171} However, the extent of ERK activation might reflect the nature or context of specific cell lines, since some cells show no or only weak activation of
ERK following cisplatin treatment. There is also conflicting evidence for the role of ERK in influencing cell survival of cisplatin-treated cells. Some studies have suggested that ERK activation is associated with enhanced survival of cisplatin-treated cells. Such is not always the case, however. Although inhibition of cisplatin-induced ERK activity by treatment with the MEK1 inhibitor PD98059 sensitizes the human melanoma cell line C8161 to cisplatin-induced apoptosis, PD98059 does not potentiate cisplatin-induced apoptosis in three other human melanoma cell lines. Instead, PD98059 protects against cisplatin-induced cytotoxicity in one of these cell lines, partly via an enhancement of cisplatin-induced of NFκB activation. Moreover, inhibition of cisplatin-induced ERK activity by PD98059 results in decreased levels of p53, p21WAF1, GADD45 and Mdm2. In addition, elevated expression of Ras, an upstream component of the ERK signaling pathway, has been connected with enhanced sensitivity to cisplatin. HeLa cell variants selected for cisplatin resistance show reduced activation of ERK following platinum treatment. This discrepancy suggests that the relationship between the activity of ERKs and the cellular response to cisplatin may depend upon individual cellular context and levels of stress.

JNK Pathway

The mechanisms of JNK1 activation in response to UV irradiation and cisplatin treatment have important differences. UV light is able to induce
translocation of JNK1 from the cytoplasm to the nuclear compartment, whereas cisplatin does not. Although a number of studies have established that JNK is activated in response to cisplatin treatment, a role for this signaling pathway in determining survival is far from clear. Several studies have provided evidence that the JNK pathway contributes to cisplatin-induced apoptosis, whereas others have suggested that JNK activation in response to cisplatin signals a protective response and is important for cell survival. Such differential effects observed from one study to another could reflect the nature of different cell types and specificity or transient vs persistent activation patterns, respectively. Interestingly, one study showed that the duration of JNK induction may be the crucial factor in mediating the signaling decision resulting in either cell proliferation or apoptosis.

**MKP-1/CL100**

Expression of phosphatase MKP-1/CL100 inhibits JNK and p38 activation after cisplatin treatment and correlates with an increase in survival after drug treatment. JNK1 induction by cisplatin is delayed and persistent, whereas trans-DDP induces JNK in a transient manner. This difference in duration of JNK induction may be due to the differential ability of inducing of MKP-1/CL-100. trans-DDP efficiently induces MKP-1/CL100, and its expression correlates with JNK inactivation, whereas cisplatin is only a very weak inducer of MKP-1/CL100. Moreover, ectopic expression of a catalytically inactive mutant of
MKP-1/CL-100 in 293 cells increases the duration of JNK activation by trans-DDP and increases toxicity of both cisplatin and trans-DDP.\textsuperscript{189,190} Overexpression of MKP-1 induces resistance to Fas-ligand triggered apoptosis in human prostate cancer cells.\textsuperscript{191} These results suggest that the persistent activation of JNK in response to cisplatin may be due to its inability to induce the expression of MKP-1/CL-100 and inhibition of MKP-1/CL100 activity in tumor cells might improve the therapeutic response to cisplatin in human cancer.

From the foregoing brief summary it is clear that cisplatin treatment of cells triggers or alters numerous defined pathways. Deciding which to apply in designing new cancer treatment strategies is critical in any chemogeno-therapeutic approach. Our recommendation is to attack those processes that occur closest in time to the formation of DNA adducts and lead most rapidly to irreversible mechanistic steps. Interruption of NER and potentiation of transcription inhibition are two such activities and, consequently, a focus of much attention in our own laboratory.

**DNA Repair Pathways**

DNA repair plays a significant role in modulating cisplatin cytotoxicity. Nucleotide excision repair, the major pathway responsible for removal of cisplatin-DNA adducts, is composed of several steps including recognition of DNA lesion, excision and removal of ~30-bp single-stranded DNA fragments containing the lesion, and re-synthesis and ligation of the newly-synthesized
repair patch to the preexisting strand.\textsuperscript{192} There are two distinct sub-pathways of nucleotide excision repair, transcription-coupled repair (TCR) and global genomic repair (GGR). TCR refers to the preferential repair of transcribed strands of RNA polymerase II-transcribed active genes, whereas GGR refers to repair throughout the genome.\textsuperscript{193,194} In human cells, the lesions are recognized by different sets of proteins for GGR and TCR; XPC/hHR23B is involved in GGR,\textsuperscript{195} whereas the Cockayne syndrome genes, CSA and CSB, are involved in TCR.\textsuperscript{196} After the different initial recognition, the two sub-pathways share common subsequent repair events; XPA and RPA are recruited and act as another damage-recognition complex that may participate in lesion verification. TFIIH is then recruited. This protein complex is involved both in transcription and in DNA repair. XPB and XPD, the component helicases of TFIIH, unwind the DNA duplex that surrounds the DNA adduct, prior to the incision step. Subsequently, XPF-ERCC1 and XPG, two structure-specific endonucleases, incise on the 5' and 3' sides of the DNA adduct, respectively, to remove a 24- to 32-base oligonucleotides containing the lesion.\textsuperscript{197} These steps are irreversible.

Cisplatin-DNA adducts are removed primarily by the NER pathway in vitro and in vivo.\textsuperscript{43,198-200} It has been suggested that the favorable clinical response of testicular cancer to cisplatin reflects its intrinsically low capacity for removal of cisplatin-DNA adducts and low levels of nucleotide repair proteins XPA, ERCC1 and XPF.\textsuperscript{201} These observations are consistent with the notion that
modification of DNA by cisplatin is the major mechanism of its cytotoxicity and that the dominant cisplatin lesions are primarily corrected by the NER pathway.

Human cells lacking functional p53 exhibit partial deficiency in GGR. The ability of p53 to modulate GGR is mediated through its ability to control the expression of p48 (also called DDB2). BRCA1 upregulates p48 expression in a p53-dependent manner following cisplatin treatment. p48 is involved in DNA damage recognition that is missing in most XPE cell lines and in CHO cells. In addition, GADD45, a protein downstream of p53, plays a role in GGR, coupling chromatin assembly and DNA repair. Overexpression of GADD45 protects cells from cisplatin, whereas inhibition of GADD45 expression by antisense DNA results in GGR deficiency and increased cell sensitivity to cisplatin. On the other hand, the role of p53 in TCR is somewhat controversial. Some laboratories reported that p53 mutant cells retained the normal TCR activity, whereas others showed that p53 also functions in TCR. This discrepancy may reflect differences in damage type and the method used to monitor repair.

The mismatch repair (MMR) process involves the recognition of base pair mismatches or other DNA damage and assembles a multimeric complex that coordinates subsequent repair events. The MMR pathway also activates other cell-cycle regulators such as p53, p73 or c-Abl, mediating the induction of cell cycle arrest and apoptosis. Cisplatin-induced activation of c-Abl depends upon functional MSH2 and MLH1. MLH1-defective HCT-116 cells and MSH2-
defective HEC-59 cells fail to activate c-Abl following cisplatin treatment; and, cisplatin activation of c-Abl kinase activity is re-established after restoration of a functional MMR in HCT-116 or HEC-59 cells. Moreover, p73 induction and apoptosis by cisplatin are also MMR and c-Abl dependent. The induction of p73 by cisplatin is abrogated in Mlh1-null and Abl-null mouse embryo fibroblasts (MEFs) but not in wild-type or Tp53-null MEFs. Both Mlh1-null and Abl-null MEFs show reduced sensitivity to cisplatin killing.

Two models have been proposed for how MMR induces cell death. One model envisions futile cycles of repair whereas the other invokes direct signaling of apoptosis. In the first model, MMR of mismatches containing cisplatin-DNA guanine (G) adducts leads to the removal and resynthesis of the paired C base in the newly synthesized strand instead of removing the cisplatin-DNA lesion, ultimately inducing cell death. In the second model, recognition of DNA damage by MMR proteins serves as a lesion-sensor and initiates a signal transduction cascade through signal transducer ATM/ATR, activating the cell cycle checkpoint and apoptosis.

However, a direct connection between the MMR pathway and cisplatin cytotoxicity has not been established. Several observations suggest that MMR deficiency is associated with low-level cisplatin resistance. In human ovarian tumor cell lines selected for cisplatin resistance, the resistance phenotype was often found to be correlated with a reduction or loss of expression of MSH2 or MLH1. There are notable exceptions, however. Although Msh2-/-
deficient cells treated with cisplatin in vivo exhibit a reduction in cell death, no
difference was occurs in the mutation rates of MMR-proficient and -deficient
cells after cisplatin exposure. Moreover, another in vivo study using
immuno-histochemical staining for hMLH1 and hMSH2 revealed no association
between MMR protein expression and overall survival of ovarian cancer patients
treated with cisplatin. The most convincing evidence comes from a recent
study in which the Cre-lox system was applied to create a cell line in which the
Msh2 gene can be inactivated de novo and then reactivated to test the contribution
of MMR to the cytotoxicity of cisplatin. With this system, no changes in the
sensitivity to cisplatin were observed upon de novo inactivation of Msh2 cell lines.
In addition, the cisplatin-resistant subclones from the freshly generated MMR-
deficient cell line could not be derived. Therefore, no evidence for direct
involvement of MMR deficiency in cisplatin resistance was found in ES cells.
The discrepancy may result from the lack of specific genetic changes that are
needed, in addition to MMR deficiency, to affect cisplatin cytotoxicity. For
example, one report suggested that defective MMR was only a minor contributor
to cisplatin resistance, accounting for less than 1.3-fold of observed cellular
resistance, and that loss of p53 response was the main determinant of
resistance. Alternatively, alterations related to DNA protection and cell cycle
progression after drug damage have been also proposed as resistance
modulators instead of MMR failure in one study using human ovarian carcinoma
cells.
Cell Death Pathways: Apoptosis and Necrosis

Cisplatin induces necrosis and apoptosis, two different modes of cell death (Figure 1.6). Necrosis is characterized by a cytosolic swelling and early loss of plasma membrane integrity. In contrast, early features of cells undergoing apoptosis include cell shrinkage, loss of attachment to the monolayers, chromatin condensation, and DNA fragmentation. The mode of cell death induced by cisplatin is concentration dependent. Primary cultures of mouse proximal tubular cells undergo necrotic cell death over a few hours after treatment with high concentrations of cisplatin (800 μM), whereas cells undergo apoptosis following exposure to only low concentrations (8 μM) of the drug over several days. PARP has been very recently identified as a novel target for cancer therapy. Excessive DNA damage induces hyper-activation of PARP. PARP cleaves the NAD$^+$ and transfers ADP-ribose moieties (ADPR) to carboxyl groups of nuclear proteins, thereby causing NAD$^+$/ATP depletion and resulting in necrotic cell death if ATP depletion reaches lethal-inducing levels.

Apoptosis, or programmed cell death, is the main response of cells to chemotherapeutic agents. Apoptosis results from activation of members of the caspase family of aspartate-specific proteases. Upstream initiator caspases such as caspase-8 and caspase-9 are activated early in the apoptotic process and they then activate other downstream caspases, caspase-3 and caspase-7. The latter are
largely responsible for cleavage of many other cellular proteins, leading to apoptosis.\textsuperscript{221}

Two major distinct apoptotic pathways have been described for mammalian cells. One involves caspase-8, which is recruited by the adapter molecule Fas/APO-1-associated death domain protein to death receptors upon extracellular ligand binding.\textsuperscript{227} The other involves cytochrome c release-dependent activation of caspase-9 through Apaf-1.\textsuperscript{228} Increased levels of cytochrome c are observed in the cytoplasm of cisplatin-treated cells, suggesting that cytochrome c release is required for cisplatin-induced apoptosis.\textsuperscript{229}

Caspase-3 plays an essential role for procaspase-9 processing and cisplatin-induced apoptosis. Cytochrome c- and caspase-8-mediated procaspase-9 processing are highly dependent on caspase-3.\textsuperscript{230-232} The apoptosis of caspase-3-deficient MCF-7 breast cancer cells is defective in response to cisplatin treatment. Stable transfection of CASP-3 cDNA into MCF-7 cells results in increased apoptosis after cisplatin treatment.\textsuperscript{232} Some studies also reported that cisplatin-induced apoptosis has caspase-3 independent pathways in cisplatin-resistant and sensitive human ovarian cancer cell lines.\textsuperscript{233} Another study showed that at least 50\% of cisplatin-induced RPTC apoptosis is independent of p53 and caspases 3, 8, and 9.\textsuperscript{234} Moreover, cisplatin can also up-regulate Fas and Fas ligand (FasL) proteins.\textsuperscript{227}

In addition, cell density could also be a factor that modulates cisplatin cytotoxicity. Recently it was reported that, at high cell density, there is a separate
cell-interdependent pathway of cisplatin killing in which damaged cells can transmit a death signal to neighboring cells. This signal is produced within the damaged cell by Ku/DNA-dependent protein kinase signaling transduced through gap junctions.\textsuperscript{235} However, another study reported that p53 transcriptional activation by DNA damage and subsequent p53-dependent apoptosis is attenuated at high cell density, possibly through modulation of cell-cell and cell-matrix interaction.\textsuperscript{236} Such molecular mechanisms remain to be elucidated. Nevertheless, the modulation by high cell density of cisplatin cytotoxicity and cellular processing pathways is a subject of significant interest that clearly warrants further investigation.

**Cisplatin and Nucleosomes**

In the eukaryotic cell, DNA is packaged in a chromatin context. The basic unit is the nucleosome, in which the DNA is wrapped around a histone octamer. The nucleosome modulates a variety of biological processes, such as replication, transcription, and repair. Incorporation of DNA into chromatin alters the dynamic and structural properties of the DNA. Therefore, nucleosome context must be taken into consideration in order to understand fully the mechanism of cisplatin cytotoxicity in vivo.

The influence of chromatin structure on cisplatin-DNA adduct formation has been investigated both in vitro and in vivo. Studies showed DNA in the linker region of polynucleosomes to be a preferred site of cisplatin DNA
binding, but this apparent preference is no longer apparent at higher drug levels. The preference for linker DNA might be attributed to histone-induced constraints on the core DNA. In addition, some studies also reveal an influence of chromatin proteins on the extent and sequence specificity of cisplatin-DNA binding. For example, enhanced damage in intact cells occurs at CACC sequences where a member of the Sp1 family of proteins is thought to bind, presumably by bending the DNA double-helix such that enhanced cisplatin binding occurs. Moreover, cisplatin and trans-DDP show different nucleosome binding preferences. Cisplatin targets the DNA, whereas trans-DDP preferentially targets the histones.

Cisplatin modification influences inter-nucleosomal DNA-protein interactions. The inhibition of micrococcal nuclease digestion rate and the change of digestion profile of cisplatin-modified chromatin suggest that cisplatin-DNA adducts alter DNA-protein interactions associated with the higher order structure of chromatin. On the other hand, micrococcal nuclease digestion indicates that cisplatin binding does not significantly disrupt the structure within the core particle, since for cisplatin modified nucleosome cores there is little effect on the digestion rate and the relative distribution of DNA fragments produced by enzymatic cleavage. DNase I digestion assays also reveal no detectable change in the DNA-protein interactions upon DNA modification.

Nucleosome structure and DNA accessibility can be altered by several multiprotein remodeling complexes or by covalent post-translational
modification of histones. Pretreatment with TSA or SAHA, two histone deacetylase inhibitors, increased the killing efficiency of cisplatin, which indicates that relaxation of the chromatin structure by histone acetylation increases the cytotoxicity of the drug.\textsuperscript{245} Similarly, the combination of arginine butyrate and cisplatin resulted in a concentration-responsive increase in cisplatin-DNA adduct formation in PC-3 cells and an overall increase in cisplatin-DNA adduct formation in three other human cancer cell lines and a significant increase in cytotoxicity of cisplatin.\textsuperscript{246} These results suggest that chromatin configuration can affect cisplatin adduct formation and modulate cytotoxicity.

With histones present, both \textit{trans}-DDP and cisplatin inhibit the reconstitution of nucleosomes. Platinated DNA, however, can be successfully incorporated into nucleosome core particles.\textsuperscript{238} These results suggest that platination of histones impedes reconstitution of free DNA, and that cisplatin may have an inhibitory role on new chromatin assembly in vivo. In addition, one study reported that cisplatin, not \textit{trans}-DDP, significantly inhibits chromatin remodeling and transcription factor binding, as well as transcription from mouse mammary tumor virus promoter in vivo.\textsuperscript{247} In addition, recent work from our laboratory using DNA alone, nucleosomes prepared with native histone, and nucleosomes prepared with recombinant, unmodified histones, each containing the same site-specific platinum-DNA adduct, reveals that the nucleosome
significantly inhibits NER. Moreover, post-translational modification of histones can modulate NER from damaged chromatin in vitro.\textsuperscript{198}

**Future Directions**

An impressive body of knowledge of the cellular processing of cisplatin has emerged in recent years, which has given us a much more detailed understanding of how cisplatin-DNA damage is recognized, how the damage signals are transduced, how the cell cycle arrests, and how DNA repair and apoptosis are activated. This knowledge provides us with an important basis and new insights to develop improved combined therapeutic strategies through chemogenotherapy and/or rational design of new platinum-based compounds. Disruption of certain pathways by inhibitors, activators or in combination with drugs that modulate cellular sensitivity to cisplatin are likely to lead to clinical benefit. DNA microarray, RNA interference, pharmaceutical intervention as well as other combined biochemistry and genetic methods will provide us with powerful tools for discovery of new proteins or pathways involved in the signaling transduction network of cellular response to cisplatin. We can anticipate more exciting discoveries in this field in coming future and, with the commitment and backing of the pharmaceutical industry, a new generation of platinum-based anticancer drugs should emerge.
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Figure 1.1. Cisplatin–DNA adducts cause a wide variety of cellular responses. The platinum atom of cisplatin binds covalently to the N7 position of purines to form 1,2- or 1,3-intrastrand crosslinks, and interstrand crosslinks. Cisplatin-DNA adducts cause a variety of cellular responses such as replication arrest, transcription inhibition, cell cycle arrest, DNA repair, apoptosis.
Figure 1.2. Mechanisms of cisplatin uptake and efflux. In addition to passive diffusion, cisplatin is also actively imported by the copper transporter Ctr1p. Copper-transporting P-type adenosine triphosphate (ATP7B) plays a role in cisplatin efflux. Both copper and cisplatin have the ability to reduce each other’s uptake and can trigger the degradation and delocalization of Ctr1p. Moreover, copper and cisplatin also exhibit directional cross-resistance.
Figure 1.3. HMGB1 protein has multiple roles. HMGB1 recognizes cisplatin-damaged DNA and modulates NER efficiency in vitro. Moreover, HMGB1 has been connected with RAG1/2, p53, MutSα. HMGB1 has been connected to MMR, V(D)J recommendation, p53 and MAPK pathways. It also facilitates nucleosome remodeling and serves as a cytokine, being secreted by necrotic and immune cells.
Figure 1.4. The p53 pathway can partially mediate cisplatin cytotoxicity. p53 is linked to DNA repair, cell cycle arrest and apoptosis. Upon DNA damage, p53 is activated and subsequently trans-activates different sets of downstream target genes, which in turn induce a variety of cellular responses.
Figure 1.5. Cisplatin activates MAPK pathways. There are three major mammalian MAPK subfamilies: ERK, JNK and p38 MAPK. Cisplatin triggers the activation of the ERK, JNK and p38 MAPK cascades in tumor cells or transformed cell lines.
Figure 1.6. Cisplatin induces necrosis and apoptosis, two different modes of cell death. DNA damage arrests the cell cycle, inhibits transcription and initiates apoptosis. Excessive DNA damage induces hyper-activation of PARP. PARP cleaves NAD⁺ and transfers ADP-ribose moieties (ADPR) to carboxyl groups of nuclear proteins. It thereby causes NAD⁺/ATP depletion, resulting in necrotic cell death if ATP depletion reaches lethal-inducing levels.
Chapter 2 Nucleotide Excision Repair from a Site-Specifically Platinum-Modified Nucleosome*

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ABBREVIATIONS

NER, nucleotide excision repair; CHO, Chinese hamster ovary; XPA, xeroderma pigmentosum complementation group A; XPF, xeroderma pigmentosum complementation group F; ERCC, excision repair cross complementing; GTG-Pt, DNA probe containing a site-specific intrastrand cisplatin cross-link at a single d(GpTpG) site; GG-Pt, DNA probe containing a site-specific intrastrand cisplatin cross-link at a single d(GpG) site; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; bp, base pair(s); IE HPLC, ion exchange high performance liquid chromatography; AAS, atomic absorption spectroscopy; PAGE, polyacrylamide gel electrophoresis; TBE, Tris-borate-EDTA; BSA, bovine serum albumin.
Introduction

Cisplatin, \textit{cis-diamminedichloroplatinum(II)}, and other platinum-based drugs such as carboplatin, \textit{cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II)} (Figure 2.1), are used to treat testicular tumors as well as a variety of other cancers (1, 2). DNA is the principal cellular target of these compounds (3), the platinum atom forming covalent bonds to the N7 positions of the purine bases. The major adducts are 1,2-intrastrand and 1,3-intrastrand cross-links (4-6). Although cisplatin and carboplatin both form these identical bifunctional adducts, the relative frequency of individual platinum-DNA cross-links is quite different. For cisplatin, 1,2-intrastrand adducts comprise 50-90%, and 1,3-intrastrand 10-25%, of all DNA lesions in cultured Chinese hamster ovary (CHO) cells treated with the drug. The percentage of 1,2- and 1,3-intrastrand cross-links for carboplatin treatment of the same CHO cells is 35-50% and 30-40%, respectively, however (6). Since carboplatin is widely used in cancer chemotherapy, it is important to study both 1,2- and 1,3-intrastrand \textit{cis-diammineplatinum(II)} cross-links formed by the two compounds.

The cellular events that are triggered by platinum-DNA damage are the subject of considerable interest, and detailed knowledge of these processes could facilitate the rational design of better platinum-based drugs. For example, the platinum compounds with different ligands which block DNA repair and transcription more efficiently, will be considered as a strategy for designing new drugs. Cells deficient in DNA repair are hypersensitive to cisplatin, suggesting
that repair plays an important role in the molecular mechanism of the drug (7, 8). To date, most studies of platinum-DNA adducts have used platinated DNA substrates, with little work having been done with nucleosomal DNA (9, 10). In eukaryotic cells, however, DNA is packaged into chromatin. The fundamental unit of chromatin structure is the nucleosome, comprising a core particle and linker DNA (11). X-ray crystal structure analysis of the nucleosome core particle has revealed how the histone octamer (H2A, H2B, H3, H4)$_2$ is assembled and the wrapping of a 146 bp DNA duplex around the outside of this core as a shallow superhelix (12). Nucleosomes modulate many cellular processes, including DNA recombination, replication, transcription and repair. (11, 13-15). It is therefore of interest to investigate the effects of platinum-DNA adducts at the nucleosome level, which is more physiologically relevant, in order to achieve a full understanding of the mechanism of action of cisplatin and its analogs.

Two major classes of chromatin-modification factors that increase DNA accessibility have been recently identified. The first class, chromatin-remodeling complexes such as SWI2/SNF2, ISWI and Mi, alter histone-DNA interactions by utilizing energy released from ATP hydrolysis (16). The second class of factors alters DNA-histone interactions through covalent modification of histones, including acetylation, phosphorylation, methylation and ubiquitination (17-19).

In the present study, we address how nucleosome structure modulates nucleotide excision repair of platinum-DNA adducts. Since we wished to learn the extent to which nucleosome structure affects the excision of different types of
platinum-DNA adducts, it was necessary to prepare substrates containing specific adducts. Accordingly, two site-specifically platinated nucleosomes were prepared, each containing a single 1,2-d(GpG-Pt) or 1,3-d(GpTgG-Pt) intrastrand cis-diammineplatinum(II) cross-link. In vitro repair assays using these substrates were carried out to compare the effect of the nucleosome core on the efficiency of platinum removal from DNA. In addition, we studied platinum excision from core particles reconstituted with either native or recombinant histones in order to determine their relative activities as substrates. The results, which are described herein, reveal the first direct evidence linking nucleotide excision repair of platinated mononucleosomes with post-translational modification of histones.

Materials and Methods

**Materials.** Cisplatin was obtained as a gift from Johnson-Matthey. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Phosphoramidites and chemicals for DNA synthesis were obtained from Glen Research. γ-32P-ATP was purchased from ICN Biomedicals.

**Synthesis of Platinated Oligonucleotides.** All oligonucleotides were synthesized on a 1 μmol scale using an Applied Biosystems DNA synthesizer (Model 392) and purified by conventional methods. The platinated oligonucleotide 20-mers, 20GG-Pt or 20GTG-Pt, were prepared as described previously (20). Briefly, a 179-μL aliquot of cisplatin (0.84 mM) was mixed with a
106-μL portion of the oligonucleotide (1.50 mM) in a 1:1 ratio in 0.01 M sodium phosphate buffer (pH 6.75). The mixture was incubated in the dark at 37 °C for 12 to 14 h. The platinated oligonucleotides were purified by ion exchange HPLC and characterized by mass spectrometry, UV spectroscopy, and atomic absorption spectroscopy (AAS).

Characterization of Oligonucleotides by UV-vis Spectroscopy, AAS, and Mass Spec. DNA concentrations were determined by UV absorption at 260 nm using a Hewlett Packard 8453 UV-vis instrument. The concentrations of bound platinum were measured on a Perkin Elmer Analyst 300 atomic absorption spectrometer equipped with a Perkin Elmer Pt lumina lamp. The ratio of bound platinum to DNA was calculated by Pt concentration divided by DNA concentration. The molecular masses of platinated oligonucleotides were conformed by MIT biopolymers lab using mass spectrometry.

Preparation of Site-Specifically Modified Platinum-DNA Probes. The synthesis of DNA duplexes containing a unique, site-specific platinum-DNA adduct was performed by enzymatic ligation of three sets of complementary oligonucleotides (Figure 2.2), as previously described (20, 21). Briefly, six fragments were annealed and ligated. The ligation products were then separated by denaturing PAGE, re-annealed, and purified by non-denaturing PAGE. The resulting DNA probes contain internal labels with $^{32}$P at the 10th phosphodiester bond 5' to the d(GpG) cross-link and at the 9th phosphodiester bond 5' to the d(GpTpG) cross-link (Figure 2.2).
Cell-Free Extract Preparation. Cell-free extracts were prepared from CHO AA8 cells by the method described previously (22).

Native Histone Octamer Preparation. Native histones H2A, H2B, H3, and H4 from HeLa S3 cells were isolated and purified as reported (23, 24). HeLa cells were grown in suspension with Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum at 37 °C. Thereafter, the cells were harvested and used for whole cell extract preparation according to a published procedure (22). The remaining nuclear pellets were lysed with a glass homogenizer and washed with 0.4 M and 0.6 M NaCl solutions. The washed nuclear pellets were adsorbed onto a dry hydroxylapatite resin. Subsequently, the core histone proteins were eluted with 2.5 M NaCl.

Expression and Purification of Recombinant Histone Proteins. Expression plasmids encoding each of the four histone proteins were transformed into E. coli BL21(DE3) cells and expressed under the control of a T7 promoter. The histones were then purified by following literature procedures (25). The histone octamer was refolded and the solution was separated by gel filtration on a 16/60 Superdex 200 (Pharmacia) column. The purified histone octamer was analyzed by 18% SDS-PAGE.

Nucleosome Reconstitution and Purification. The radioactively labeled 199-bp DNA probes were assembled into nucleosomes with the histone octamer as described previously (26). A 1 pmol quantity of DNA substrate was incubated with the HeLa core histones in a 1:1 molar ratio, 1 μg of BSA, and 2 M NaCl in a
final volume of 10 μL for 15 min at 37 °C. The reaction mixture was serially
diluted by adding 3.3, 6.7, 5.0, 3.6, 4.7, 6.7, 10, 30, and 20 μL portions of 50 mM
HEPES (pH 7.5), 1 mM EDTA, 5 mM dithiothreitol (DTT), and 0.5 mM
phenylmethylsulfonyl fluoride (PMSF) at 15 min intervals over a period of 2.5 h
incubating at 30 °C. The resulting solution was reduced to 0.1 M in NaCl by
adding 100 μL of 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40,
5 mM DTT, 0.5 mM PMSF, 20% glycerol, and 100 μg/mL of BSA, and incubated
for 15 min at 30 °C. After reconstitution, the mononucleosomes were purified
from free DNA by centrifugation through an 11-mL, 5-25% sucrose gradient in
10 mM HEPES-KOH (pH 7.9)-1 mM EDTA-0.1% Nonidet P-40 using a SW41
rotor (25,000 rpm, 18 h, 4 °C) according to published methods (27).
Reconstitution products and fractions separated by sucrose gradient were
analyzed by non-denaturing polyacrylamide gel electrophoresis (6%
polyacrylamide; 1X Tris-borate-EDTA [TBE]) (28). Fractions containing
mononucleosomes were used for the excision assay, which measures the release
of 24 to 32 nt-long oligomers carrying the damage site (29, 30). The recombinant
nucleosomes were prepared in the same manner.

Excision Repair Assay. Cell-free extracts from CHO AA8 cells were used to
measure excision with the 199-bp DNA substrates in the form of a
mononucleosome or free DNA as described elsewhere (28). Briefly, in a typical
excision assay, 1.5 to 3 fmol of substrate DNA (either free DNA, native
nucleosome or recombinant nucleosome) were incubated with 50 μg of cell-free
extracts at 30 °C in 25 μL of excision repair buffer (32 mM HEPES-KOH, pH 7.9, 64 mM KCl, 6.4 mM MgCl₂, 0.24 mM EDTA, 0.8 mM DTT, 2 mM ATP, 0.2 mg/mL BSA, 5.5% glycerol, 4.8% sucrose). The reaction mixtures were incubated at 30 °C for 2 h to 4 h. The reaction products were purified by phenol-chloroform extraction and analyzed on a denaturing PAGE gel (8% polyacrylamide; 1X TBE). The extent of excision was determined by measuring the levels of radioactivity in the bands of excised products (20-30 nt range) and unexcised substrate with a phosphorimager and ImageQuant system (Molecular Dynamics).

Results

Synthesis and Purification of DNA 199GG-Pt and 199GTG-Pt Probes. The oligonucleotides were prepared and purified by PAGE and the purity was confirmed by HPLC and mass spectrometry. The platinated oligonucleotides (GG-Pt and GTG-Pt) were characterized by UV-vis spectroscopy, AAS (31), and mass spectrometry. The 199-mer was synthesized by modification of a previously described strategy (Figure 2.2) (21). The ligation products were then purified by PAGE (Figure 2.3), and the bands corresponding to the 199-mer (Figure 2.3A) were excised and eluted. The double stranded 199-mer (ds-199-mer) was purified by non-denaturing PAGE (Figure 2.3B). The total yield of ds-199-mer ranged from 1-5% based on starting material.

Reconstitution and Purification of Nucleosomes. The recombinant histone octamer was refolded and purified. The SDS-PAGE gel analysis revealed that the
individual recombinant histones and recombinant histone octamer are > 95% pure (Figure 2.4). The internally $^{32}$P-labeled DNA probes and octamer were reconstituted into nucleosomes with native or recombinant histones. The reconstituted nucleosomes were separated from free DNA by sucrose gradient centrifugation. Analysis on a non-denaturing gel (Figure 2.5) revealed that the nucleosome band migrates more slowly than its DNA component, in agreement with previous work (28). The GTG-Pt and GG-Pt modifications did not significantly alter the migration of either free or nucleosomal DNA in the gel. The presence of a sharp band in lanes 2 and 4 (Figure 2.5) indicates good substrate positioning of the nucleosomal DNA wrapped around the histone octamer.

**Excision Assay on Nucleosomal DNA.** The nucleosomal DNAs were used as substrates for in vitro nucleotide excision repair under the same conditions as for free DNA. The reaction products were analyzed by 8% denaturing PAGE (Figure 2.6A). Comparison of repair signals for free and native nucleosomal DNA containing the same cisplatin adduct clearly indicates that the nucleosome inhibits excision under our assay conditions. For the free GTG-Pt DNA substrates, the excision is about 10% ± 2% (averaged results from 4 experiments) of total DNA, whereas for the native nucleosomal GTG-Pt DNA, the excision is only 1.2% ± 0.4% (averaged results from 3 experiments). Upon changing from free GG-Pt DNA to native nucleosomal GG-Pt DNA, the excision signal decreases from 1.1% ± 0.3% (averaged results from 4 experiments) to 0.35% ±
0.14% (averaged results from 3 experiments) of total DNA. Thus, the efficiency of excision repair of nucleosomal DNA GG-Pt is about 33% of free DNA GG-Pt, whereas the efficiency of excision of nucleosomal DNA GTG-Pt is about 12% that of free DNA GTG-Pt (Figure 2.6B).

To investigate the role of histone post-translational modification in modulating NER of platinum-DNA adduct at the mononucleosome level, native histone octamers composed of post-translationally modified histones and recombinant histone octamer composed of non-modified histones were prepared and purified. In vitro nucleotide excision repair of the native and recombinant nucleosomal DNA probes were carried out under the same conditions. The reaction products were analyzed by 8% denaturing PAGE (Figure 2.7). Comparison of excision signals for native and recombinant nucleosomal DNA containing the same cisplatin adduct clearly indicates that the efficiency of NER of native nucleosomal DNA GTG-Pt is 2.5 ± 0.4 fold higher than that of recombinant nucleosomal DNA GTG-Pt. Similar stimulation of NER (2.2 ± 0.4 fold) was also observed from native nucleosomal GG-Pt, compared to that of recombinant nucleosome GG-Pt (data not shown).

Discussion

In this work we prepared site-specifically platinated mononucleosomes and compared the relative excision of platinum damage from free versus
nucleosomal DNA, and from nucleosomes reconstituted with native versus recombinant histone core proteins.

Comparison of NER from Free vs Nucleosomal DNA. This study reports the first synthesis and characterization of mononucleosomes containing site-specific cis-[Pt(NH$_3$)$_2$]$^{2+}$ d(GpG) or d(GpTpG) intrastrand cross-links. With the use of these substrates, we found the excision of damage from nucleosomal DNA to be significantly less than that from free DNA. This observation reveals that nucleosomes protect platinum-DNA adducts from being repaired. There are several possible reasons for this result. First, core histone-DNA interactions reduce the association constants for damaged DNA-binding proteins of the repair complex by tenfold (24, 28). Second, the site of damage might be shielded by the histone octamer surface or histone protein tails, thus limiting access of the repair machinery. Third, the nucleosome significantly alters DNA structure. Nucleosomal DNA is overwound by about 0.3 bp per turn compared to free DNA (12). Finally, the distortion caused by the cisplatin adducts on nucleosomal DNA might differ from that produced by the same adduct on free DNA, thus lowering the overall recognition efficiency of damage sensor proteins. Taken together, these effects can limit the ability of NER proteins to access or recognize the platinum cross-link owing to a modulation of nucleosome structure. This result is consistent with the previous studies of UV- or AAF-damaged nucleosomes (24, 28, 32, 33).
Our results are also in accord with the observation that repair in actively transcribed genes is more rapid and efficient than in non-transcribed regions of the genome (34). We suggest that one possible factor contributing to this phenomenon of transcription-coupled repair is that, in the transcribed gene, when damage is in the template strand, DNA is more exposed compared to its tight packaging in nucleosomes comprising chromatin of inactive genes (35).

The degree of nucleosome protection differs for the GG-Pt and GTG-Pt cross-links. This difference probably reflects the degree of distortion of GG-Pt and GTG-Pt adducts. Previous structural studies reveal that DNAs containing GG-Pt and GTG-Pt lesions are strikingly different (2, 36). Gel electrophoresis experiments indicate that the 1,2-d(GpG) cisplatin adduct bends the DNA helix by 32-34° towards the major groove of DNA and unwinds the DNA helix by 13°, whereas the 1,3-d(GpTpG) adduct bends 35° and unwinds the DNA helix by 23° (37, 38). Further confirmation is provided by X-ray and NMR structural studies (39, 40). The NMR structure of a single 1,3-d(GpTpG) cisplatin adduct shows it to be more distorted than the structure of DNA containing a single 1,2-d(GpG) cross-link (41-44). The central T is extruded into the minor groove and base pairing is lost at the 5' G and central T/A base pair (44). These significantly different structural distortions between 1,2- and 1,3-intrastrand cross-links are already known to produce differential recognition and processing by cellular proteins (45). The nucleosome modulation adds another level of complexity, the
detailed nature of which must await the availability of structural information on site-specifically platinated nucleosomes.

_Comparison of NER from Nucleosomes with Native vs Recombinant Histones._ Repair synthesis of UV-damaged DNA is enhanced within nucleosome cores of hyperacetylated chromatin in butyrate-treated human cells (46). The origin of enhanced repair synthesis was not identified, however, and could have arisen from a greater population of minor UV photoproducts, such as pyrimidine-pyrimidone (6-4) dimers, in the highly acetylated nucleosomes. The substrates employed in this study were globally modified and then digested to mononucleosomes. Since the DNA sequence differs for each mononucleosome, the types, the amount, and the locations of UV damage are also variable. Thus it is difficult to evaluate the effects of post-translational modification on a specific type of DNA damage or location within the nucleosome.

The present study, which reports the first reconstituted, site-specifically platinated mononucleosome containing either recombinant or native, and therefore post-translationally modified histones, provides a powerful tool to investigate these above questions. In our biochemically well-defined system, the excision efficiency of the native nucleosomes is significantly greater than that of recombinant nucleosomes, revealing that overall post-translational modification of histones stimulates NER in this context.

There are at least three possible reasons for this effect. First, histone modification changes the nucleosome structure and surface environment, and
thus can improve the accessibility of nucleosomal DNA to the repair apparatus. Acetylation occurs on lysine residues at the basic, N-terminal tail domains of the core histones. One consequence of acetylated N-terminal tails is a reduced affinity for DNA owing to charge neutralization, thus destabilizing chromatin structure (for reviews, see (47, 48)).

Second, the histone modification can recruit remodeling factors. Transient histone hyperacetylation acts as a signal for ATP-dependent remodeling complexes at the PHO8 promoter in vivo (49). Moreover, acetylation of histone H4 K8 mediates recruitment of the SWI/SNF complex to DNA (50), and it was recently reported that SWI/SNF can stimulate excision repair of human excision nuclease in the mononucleosome core particle, made with post-translationally modified histones, by increasing DNA accessibility (24). Given these findings, we conclude that histone covalent modifications, nucleosome remodeling and nucleotide excision repair are linked processes.

Third, histone modification also enlists proteins involved in NER. Access of DNA repair machinery to UV lesions within chromatin is facilitated by TBP-free-TAFII complex (TFTC) via covalent modification of chromatin. A subunit of TFTC (SAP130) shares homology with the large subunits of UV-damaged DNA binding factor (DDB). TFTC is recruited to UV damaged DNA in parallel with the nucleotide excision repair protein XP-A. The fact that TFTC can recognize UV-damaged DNA and preferentially acetylate nucleosomes assembled on UV-irradiated DNA suggests a possible role for TFTC in making the DNA damage
accessible for repair in the context of chromatin (51). In addition, CREB binding protein (CBP) and p300 histone acetyl-transferases can interact with the small subunit of XP-E damage-specific DNA binding protein (DDB) (52). Taken together, these findings may indicate that selective acetylation of histones on damaged nucleosomes may provide a general strategy for recruiting NER factors more efficiently to overcome the nucleosome barrier to excision repair. These three possibilities are not mutually exclusive. Our results are consistent with the previous studies of the function of histone post-translational modification and ability to stimulate transcription (53-55). It is likely that cells utilize post-translational modifications as a signal or factor, through some common mechanism, to regulate NER and transcription on chromatin.

The relative stimulation of NER by post-translationally modified histones is similar for both GG-Pt and GTG-Pt cross-links. This result indicates that histone modification is likely to affect overall nucleosome structure in a general rather than adduct-specific manner.

The present experiments were carried out at the mononucleosome level in vitro. Histone modifications can significantly alter higher order nucleosome packing, however, and thus change the accessibility of nucleosome to NER (for a review, see (17, 18)). We suggest that post-translational modification of histones will have an important effect on NER in vivo, as has already been demonstrated for transcription regulation (53-55).
Conclusion. With the use of site-specifically platinated DNA in mononucleosomes, we determined that nucleotide excision repair in mammalian cell extracts is substantially diminished compared with free DNA containing the same adducts. A comparison of the extent of repair of native and recombinant nucleosome substrates revealed excision from native nucleosomal DNA to be about 2-fold higher than the level observed with recombinant, unmodified nucleosomal DNA. This result indicates that post-translational modification of histones can play a key role in modulating nucleotide excision repair of platinum-DNA adducts in chromatin. The relative effect of the nucleosome does not depend on the nature of the adduct. The in vitro system we established in this study will facilitate investigation of other cellular processing of platinum-DNA damage and help to elucidate the role of specific post-translational modifications in NER of platinum-DNA adducts at the nucleosome level.

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References


Figure 2.1. Structures of cisplatin and carboplatin. A: cisplatin, cis-diammine-dichloro(platinum(II). B: carboplatin, cis-diammine(1,1-cyclobutanedicarboxylato)(platinum(II).
Figure 2.2. Strategy for synthesizing site-specifically platinated DNA probes. Six synthetic oligonucleotides, one bearing the platinum intrastrand cross-link and a 5'\(^{32}\)P radiolabel denoted by an asterisk, were annealed and ligated as shown. Sequences of the individual strands are depicted.
Figure 2.3. (A) Denaturing PAGE gel of ligation products of site-specifically platinated DNA probes. Lane 1: ligation reaction of 199-mers DNA GG-Pt; lane 2: ligation reaction of 199-mer DNA GTG-Pt. The 199-mers are shown by the arrow. (B) Non-denaturing PAGE gel of purified site-specifically platinated ds-DNA probes. Lane 1: ds-199-mer DNA GG-Pt; lane 2, ds-199-mer DNA GTG-Pt; lane 3, 25 bp DNA ladder; lane 4, 100 bp DNA ladder.
Figure 2.4. SDS-PAGE gel of recombinant histones and histone octamer. Lane 1, H2A; lane 2, H2B; lane 3, H3; lane 4, H4; lane 5, octamer.
Figure 2.5. Non-denaturing PAGE gel of nucleosome and free DNA. Lane 1: free 199GG-Pt DNA; lane 2: nucleosomal 199GG-Pt DNA; lane 3: free 199GTG-Pt DNA; lane 4: nucleosomal 199GTG-Pt DNA.
Figure 2.6. (A) Excision assay with nucleosomal and free DNA. Lane 1: nucleosomal 199GG-Pt DNA; lane 2: free 199GG-Pt DNA; lane 3: NER nucleosomal 199GTG-Pt DNA; lane 4: free 199GTG-Pt DNA. The numbers the bottom of each lane are the excision percentages of the repair reactions. (B) Efficiency of excision of cisplatin lesions from nucleosomal DNA. The efficiency of excision of nucleosomal DNA GTG-Pt is about 12% of free DNA GTG-Pt, whereas the efficiency of nucleosomal GG-Pt is about 33% of free DNA GG-Pt.
Figure 2.7. Analysis of repair of native and recombinant nucleosomal platinated DNA by the excision assay. Lane 1: native nucleosomal 199GTG-Pt DNA; lane 2: recombinant nucleosomal 199GTG-Pt DNA. The efficiency of excision from native nucleosomal DNA was 5.3% (lane 1) and from nucleosomes reconstituted from recombinant histones was 2.2% (lane 2). The absolute per cent efficiency in lane 1 cannot be compared with that in lane 3 of Figure 2.6 because of different incubation times, buffer conditions, and batch of cell extract.
Chapter 3  Nucleotide Excision Repair of Site-Specifically Platinum-Modified Tetrasomes
Introduction

The nucleosome, the basic structural unit of chromatin, is composed of a histone \((H3/H4)_2\) tetramer flanked by two H2A/H2B dimers, around which is wrapped 146 base pairs (bp) of DNA.\(^1\) The histone octamer is maintained by hydrogen bonds between the \((H3/H4)_2\) tetramer and the two H2A/H2B dimers.\(^2\) The interaction of the H2A/H2B dimer with the \((H3/H4)_2\) tetramer is significantly weaker than the interaction between histones H3 and H4 within the tetramer.\(^2,3\) An H2A/H2B dimer exhibits different dynamic behavior compared to the \((H3/H4)_2\) tetramer, being more easily released and exchanged from the octamer in vivo and in vitro.\(^4-6\) In addition, the turnover rate of H2A and H2B is also faster than that of H3 and H4.\(^7-9\)

The \((H3/H4)_2\) tetramer plays a central role in maintaining the structural integrity and positioning of the nucleosome core particle.\(^10-12\) The tetrasome particle, formed by a histone \((H3/H4)_2\) tetramer and DNA, is similar in overall dimensions to the entire nucleosome core particle. The full 1.75 superhelical turns of DNA can be maintained. The shielding effects of the \((H3/H4)_2\) tetramer are sufficient to allow for the generation of many nuclease resistant fragments that are characteristic of complete nucleosomes.\(^13\) Nuclease digestion results reveal that the \((H3/H4)_2\) tetramer is bound to, and protects the central 70-80 bp region, of nucleosomal DNA.\(^14,15\)
During DNA replication, the H3 and H4 histones together behave as an entity that is distinct from histones H2A and H2B. (16-18) Newly synthesized H3 and H4 deposit as a tetramer and associate with old H2A and H2B. (18) The \((H3/H4)_2\) histone tetramer interacts specifically with chromatin assembly factors and chaperones, which are required for nucleosome assembly at DNA replication forks. (19,20) Moreover, histones H3 and H4 together with ASF1 (anti-silencing function 1 protein) assemble into the RACF complex (replication-coupling assembly factor), which mediates the assembly of newly synthesized DNA into chromatin during DNA replication and the repair of UV damaged DNA. (19,21)

The effects of the \((H3/H4)_2\) tetrasome on transcription depend upon the nature of the RNA polymerase. Both tetrasomes and nucleosomes can serve as absolute blocks of transcription elongation by RNA polymerase II, even in the presence of elongation factors such as TFIIS in vitro. (22) On the other hand, the tetrasome and nucleosome core particles exhibit different effects on T7 RNA polymerase, a much simpler transcription system. The histone \((H3/H4)_2\) tetramer has little effect on transcription initiation, thus allowing efficient transcription in vitro by T7 RNA polymerase, whereas the histone octamer causes transcriptional inhibition mainly by blocking initiation. (15)

The presence of the nucleosome also serves as a barrier for DNA repair. Previously we reported that nucleosomes significantly inhibit nucleotide excision repair (NER) of cisplatin-modified DNA in vitro, (23) however, little is known
about the effect of the tetrasome on such repair. It is of interest to learn whether histone tetramers are also sufficient to block the repair of cisplatin-DNA adducts. In the present chapter, we describe the results of our studies of this process. In addition, the \([\text{Pt(DACH)}]^2\text{+}\)-modified tetrasomes were also prepared to evaluate how changes in the spectator ligands on platinum might affect excision repair at the tetrasome level.

### Method and Materials

**Materials.** Cisplatin was obtained as a gift from Engelhard. Reagents for DNA synthesis were purchased from Glen Research. SequaGel diluent and concentrate solutions for preparing denaturing polyacrylamide gels were purchased from National Diagnostics. T4 polynucleotide kinase, T4 DNA ligase, and NEB buffer 3 were obtained from New England Biolabs. \(\gamma^{32}\text{P}-\text{ATP}\) was purchased from Perkin-Elmer.

**Synthesis of Platinated Oligonucleotides.** All oligonucleotides were synthesized on a 1 \(\mu\text{mol}\) scale with an Applied Biosystems DNA synthesizer (Model 392) and purified by conventional methods. The platinated oligonucleotides were prepared, purified and characterized as described previously.(23)

**Preparation of Site-Specifically Modified Platinum-DNA Probes.** The synthesis of blunt-ended DNA duplexes containing a unique, site-specific platinum-DNA adduct was performed by enzymatic ligation of complementary oligonucleotides
(Figure 3.1), as previously described. (24,25) Briefly, five oligonucleotides fragments were phosphorylated using T4 polynucleotide kinase and ATP (γ-32P-ATP, where appropriate). All the oligonucleotides were annealed and then incubated with T4 DNA ligase at 16 °C for 16 h. The ligation products were separated by denaturing PAGE. The band corresponding to the desired product was excised from the gels. The DNA was eluted, re-annealed, and separated by non-denaturing PAGE. The resulting DNA probes contain internal labels with 32P at the 7th phosphodiester bond 5' to the 1,3-d(GpTpG) cross-link (Figure 3.1).

**Cell-Free Extract Preparation.** Cell-free extracts were prepared from CHO AA8 cells by the method described previously. (26)

**Expression and Purification of Recombinant Histone Proteins.** The four histone proteins were expressed under the control of a T7 promoter in E. coli BL21(DE3) cells and purified following published procedures. (27,28) Purified histones were mixed with appropriate molar quantities (equimolar amounts of H2A:H2B:H3:H4 for the octamer; for the tetramer, H3:H4 = 1:1) in 7 M guanidinium HCl, 20 mM Tris-HCl (pH = 7.5), and 10 mM DTT. The histone protein solution was dialyzed against refolding buffer (2 M NaCl, 10 mM Tris-HCl (pH = 7.5), 1 mM Na-EDTA, 2 mM DTT) at 4 °C through three changes over a period of 48 h. The dialyzed histone octamer and/or tetramer solution were then separated by gel filtration on a 16/60 Hi-Load Superdex 200 column (Pharmacia). The purified histone octamer and tetramer were analyzed on a 4-20% Tris-HCl SDS-PAGE.
Reconstitution and Purification of the Tetrasome and Nucleosome. The $^{32}$P radioactively labeled DNA probes were assembled into either nucleosome or tetrasome with the histone octamer or tetramer, respectively, in the manner described previously.(23) The nucleosome and tetrasome products were then purified from free DNA by centrifugation through an 11-mL, 5-25% sucrose gradient in 10 mM HEPES-KOH (pH = 7.9), 1 mM EDTA, 0.1% Nonidet P-40 using a SW41 rotor (35,000 rpm, 18 h, 4 °C). The purified nucleosome and tetrasome products were used as substrates in an in vitro nucleotide excision repair assay.

Excision Repair Assay. Cell-free extracts from CHO AA8 cells were used to measure excision with the DNA substrates in the form of a nucleosome, tetrasome, or free DNA as described previously.(23) Briefly, in a typical excision assay, 1.5 to 3 fmol of substrate DNA (either free, tetrasomal, or nucleosomal DNA) were incubated with 50 μg of cell-free extracts at 30 °C in 25 μL of excision repair buffer (32 mM HEPES-KOH (pH = 7.9), 64 mM KCl, 6.4 mM MgCl$_2$, 0.24 mM EDTA, 0.8 mM DTT, 2 mM ATP, 0.2 mg/mL BSA, 5.5% glycerol, 4.8% sucrose). The reaction mixtures were incubated at 30 °C for 3 h. The reaction products were purified by phenol-chloroform extraction and analyzed on a denaturing PAGE gel (8% polyacrylamide; 1X TBE). The extent of excision was determined by measuring the levels of radioactivity in the bands of excised products (24-32 nt range) and unexcised substrate with a phosphorimager and ImageQuant system (Molecular Dynamics).
Results and Discussion

The histone octamer and (H3/H4)$_2$ tetramer was prepared, purified and analyzed on a 4-20% Tris-HCl SDS-PAGE (Figure 3.2). The two bands in lane 6 (histone tetramer) correspond to histone H3 and H4 with a 1:1 ratio.

The site-specifically cisplatin-modified DNA was synthesized and purified by 5% denaturing gel electrophoresis and subsequently on a 5% non-denaturing gel (Figure 3.3). The purified dsDNA probes were then assembled into nucleosome and tetrasome substrates and purified by sucrose gradient centrifugation (Figure 3.4). The resulting purified tetrasomes and nucleosomes were used as substrates for the in vitro nucleotide excision repair assay under the same conditions as for free platinated DNA. The reaction products were analyzed on an 8% denaturing PAGE (Figure 3.5). For the naked 1,3-d(GpTpG)-Pt DNA templates, the amount of excised product is about 16.6% ± 0.3%, determined by the ratio of the counts of the rapidly moving bands (24-32 nt) to the total counts in the lane. The excision efficiency is only 1.7% ± 0.3% and 1.6% ± 0.1%, however, for the nucleosomal and tetrasomal 1,3-d(GpTpG)-Pt DNA, respectively. The nucleosome inhibits excision to about 10% of the level observed with free DNA, which is consistent with our previous report with a 199mer DNA.(23) The presence of linker DNA does not appear to be an important factor in the inhibitory effect of the nucleosome on NER.
Comparison of the repair signals of free and tetrasomal DNA indicates that the tetrasome can also significantly inhibit excision repair of DNA, the level of inhibition being similar to that of the nucleosome. The 1,3-d(GpTpG) platinum-DNA adduct is located at the 70-72nd nucleotides from 5' terminus of the 146mer DNA, presumably interacting with the adjacent H3/H4 tetramer in the nucleosome, as revealed in the previously reported nucleosome structure.(29) The similarity in repair inhibition is therefore likely a result of the similar local environment around the damage site within the tetrasome and nucleosome.

Similar to cisplatin-modified DNA, the site-specifically 1,3-d(GpTpG) \{Pt(DACH)\}^{2+}-modified DNA and tetrasome were prepared as substrates for in vitro nucleotide excision repair. Comparison of the excision efficiency of \{Pt(DACH)\}^{2+}-modified tetrasomal and free DNA indicates that \{Pt(DACH)\}^{2+}-modified tetrasome also significantly inhibits the excision repair (Figure 3.6). The extent of excision from 1,3-d(GpTpG) \{Pt(DACH)\}^{2+}-modified DNA is 19%, whereas for the \{Pt(DACH)\}^{2+}-modified tetrasomal DNA, the excision signal decreases to 3%, or about 6-fold inhibition. In agreement with earlier reports, DNA with either \{Pt(DACH)\}^{2+} or cisplatin modification was excised at a similar level in naked DNA.(30) However, comparison of the excision efficiencies of the modified substrates reveals that of the cisplatin-modified tetrasomal DNA to be about half (53%) that of \{Pt(DACH)\}^{2+}-modified probe. The influence of 1,2-diammineplatinum(II) spectator ligand is therefore much more significant in tetrasomes than in free DNA. The presence of cyclohexane ring has only a minor
effect on the adduct geometry, even considering formation of a H-bond with a nearby guanosine, the overall structures of the cisplatin-DNA and \( \text{Pt(DACH)}^{2+} \)-DNA complexes being very similar.(31,32) Therefore, the differential repair we observe here is most likely a consequence of the interaction of the DACH cyclohexane ring and the core histones. Alternatively incorporation into the tetrasome may modulate the cisplatin and \( \text{Pt(DACH)}^{2+} \) structure differently, thus changing the repair efficiency.

Previous work revealed that the histone tetramer can protect from nuclease digestion only about 70 bp of DNA, whereas the full nucleosome blocks 146 bp. The histone tetramer thus provides similar protection as the nucleosome when the cisplatin lesion is located within the central 70 bp range. On the other hand, the tetrasome may be less able to protect the damage site when it is located beyond the central 70 bp range. Therefore, globally platinated DNA may be better repaired in tetrasomes compared with nucleosomes in vivo. Moreover, the absence of H2A/H2B dimer decreases the folding of nucleosome arrays into higher-order structures.(33) Therefore, the change in higher order chromatin structure that occurs by releasing H2A/H2B may also stimulate repair in vivo.

In addition to disassembling nucleosomes, the cell has other means by which to increase DNA accessibility hence maintain efficient transcription and repair. Two major mechanisms, covalent histone post-translational modifications and nucleosome remodeling complexes, increase the efficiency of transcription and repair from nucleosomes in vivo.(34,35) In particular, the \((\text{H3/H4})_{2}\) histone
tetramer is important in both mechanisms. The presence of the tail domains of core histones, especially those in the \((H3/H4)_2\) tetramer, inhibits transcriptional initiation. \((36,37)\) Removal of the histone H3 and H4 tails is sufficient to restore the access of nucleosomal DNA to TFIIIA and increases the transcriptional activity of the 5S nucleosomal templates in vitro. \((38,39)\) In addition, acetylation of the \((H3/H4)_2\) histone tetramer is accompanied by substantial stimulation of transcription. \((39-41)\) Histone post-translational modifications also stimulate repair of damage from the nucleosome. \((23,42,43)\) In the case of the remodeling complex SWI/SNF, which has been linked to the stimulation of transcription and DNA repair from nucleosomes, interactions of the global domain of histone H3 are crucial for SWI/SNF association with nucleosomes. \((44)\) SWI-SNF can also remodel \((H3/H4)_2\) tetrasomes, although less efficiently compared to nucleosomes. \((45)\) Therefore, releasing H2A/H2B, modifying histone tails, and remodeling by remodeling complex may act synergistically to relieve the nucleosome barrier for repair the cisplatin-DNA adducts in vivo.

In conclusion, We demonstrate that the \((H3/H4)_2\) tetramer is sufficient to block the excision of both cisplatin- and \([Pt(DACH)]^{2+}\)-DNA adducts in vitro; therefore, releasing H2A/H2B alone is not sufficient to relieve the inhibition at mononucleosome level. Cells may adopt several ways to relieve such a barrier. In addition, the tetrasome structure inhibits \([Pt(DACH)]^{2+}\) and cisplatin-modified differentially, which may be important for their different profiles of anticancer activity.
Acknowledgment

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References


A B C

Bf M

A: 63mer; B: 14GTG-Pt; C: 69mer; D: 86mer; E: 60mer.

Fragment 63mer:
5'-ATCAATATCCACCTGCAGATTCTACCAAAAGTGTATTTGGAAACTGCTCCATCAAAAGGCA
TG-3'

Fragment 14merGTG: 5'-TTCACCGTGATTCC-3'

Fragment 69mer:
5'-CCTCAACATCGGAAAACTACCTCGTCAAAGGTATGTGAAAACCATCTTAGACGTCCACC
TATAACTA-3'

Fragment 86mer:
CAC: 5'-ATGTTGAGGGGAATCACGGTGAACATGCCTTrGATGGAGCAGTTTCCAAATACACT
TTTGAGAATCTGCGAGTTGATATTGAT-3'

Fragment 60mer:
5'-TAGTTATAGGGTGGAGTGAACATCTGCTTTTGTAGGAGGGCATTCCTTCCAAATACACT
TTTGAGAATCTGCGAGTTGATATTGAT-3'

Figure 3.1. Strategy for synthesizing the blunt-ended site-specifically platinated DNA probe. Five synthetic oligonucleotides, one bearing a 1,3-d(GpTpG) intrastrand platinum-DNA cross-link and a 5'-32P radiolabel denoted by an asterisk, were annealed and ligated as shown. Sequences of the individual strands are depicted.
Figure 3.2. 4-20% SDS-PAGE gel of recombinant histones, histone (H3/H4)$_2$ tetramer and histone octamer. Lane 1, histone H2A; lane 2, histone H2B; lane 3, histone H3; lane 4, histone H4; lane 5, histone octamer; lane 6, histone (H3/H4)$_2$ tetramer.
Figure 3.3. Non-denaturing PAGE gel of purified double-stranded DNA 146-mer GTG-Pt. The site-specifically platinated DNA probe was prepared by the enzymatic ligation described in the text. The resulting products were purified by 5% denaturing PAGE gel electrophoresis and subsequently by 5% non-denaturing PAGE gel electrophoresis. Lane 1, 100-bp DNA ladder; lane 2, purified ds DNA 146-mer GTG-Pt.
Figure 3.4. Non-denaturing PAGE gel electrophoresis analysis of site-specifically cisplatin-modified tetrasome (A), \{Pt(DACH)\}^{2+}-modified tetrasome (B), and cisplatin-modified nucleosome (C). Different fractions collected from sucrose gradient centrifugation for each sample were loaded onto the non-denaturing PAGE gel and visualized by autoradiography.
Figure 3.5. Nucleosome and tetrasome inhibition of nucleotide excision repair of cisplatin-DNA adduct. The cisplatin-modified substrate, either free (lane 1), tetrasomal (lane 2), or nucleosomal DNA (lane 3), was incubated with a cell-free extract at 30 °C for 3h and products were analyzed on an 8% denaturing gel and visualized by autoradiography.
Figure 3.6. Tetrasome inhibition of excision repair of cisplatin- and [Pt(DACH)]^{2+}-DNA adduct. The substrates [Pt(DACH)]^{2+}-modified DNA (lane 1), its tetrasome (lane 2), cisplatin-modified DNA (lane 4) and its tetrasome (lane 3) were incubated with a cell-free extract at 30 °C for 3h and the products were analyzed on an 8% denaturing gel and visualized by autoradiography.
Chapter 4 Cisplatin Adducts Change the Rotational Positioning of DNA on the Nucleosome
Introduction

In eukaryotic cells, DNA is packaged into chromatin. The nucleosome, the basic structural unit of chromatin, consists of a histone octamer, which is composed of a (H3/H4): tetramer and two H2A/H2B dimers, around which is wrapped 146 bp (base pairs) of DNA. Incorporation of DNA into chromatin alters the structural and functional properties of the DNA. Nucleosomes modulate a variety of biological processes, such as replication, transcription, and repair. The rotational setting of the DNA on the nucleosome core provides an asymmetric environment for the double helix, which limits transcription factors to the bases facing away from the histone core surface.

Cisplatin, cis-diaminedichloroplatinum (II), is used to treat testicular, ovarian, cervical, head and neck, esophageal, or non-small cell lung cancer. Cisplatin enters the cell through either passive diffusion or transporter-mediated uptake. The lower intracellular chloride ion concentration (~20 mM) facilitates displacement of chloride ligand by a water molecule, transforming cisplatin into an activated, aquated form, cis-[Pt(NH3)2Cl(OH)2]+. This activated form of cisplatin reacts readily with DNA to form a covalent bond with the N(7) atom of a purine base. Cisplatin-DNA adducts significantly distort the DNA structure by unwinding the helix and bending it in the direction of the major groove.

The influence of chromatin structure on cisplatin-DNA adduct formation has been investigated both in vitro and in vivo. Cisplatin and trans-DDP show different
nucleosome binding preferences. Cisplatin predominantly targets the DNA and forms intrastrand cross-links, whereas trans-DDP preferentially forms histone-DNA cross-links. (10-12) In polynucleosomes, the DNA in the linker region is the preferred binding sites for cisplatin, (13, 14) but higher drug levels abolish this apparent preference. (12, 13, 15) In addition, the nucleosome structure has a significant inhibitory effect on the repair of cisplatin-DNA adducts. (16)

Understanding the interaction of cisplatin-DNA adducts with nucleosomes and their effects on nucleosome structure and functions can contribute to the rational improvement of the anticancer properties of the drug. Here we use hydroxyl radical and DNase I footprinting to characterize site-specifically cisplatin-modified nucleosomes. Preformed cisplatin-DNA adducts change the rotational setting of DNA with respect to the histone surface during nucleosome formation.

Materials and Method

Materials. Cisplatin was obtained as a gift from Engelhard. Reagents for DNA synthesis were purchased from Glen Research. SequaGel diluent and concentrate solutions for preparing denaturing polyacrylamide gels were obtained from National Diagnostics. T4 polynucleotide kinase, T4 DNA ligase, and DNase I were purchased from New England Biolabs. Phosphoramidites and chemicals for DNA synthesis were from Glen Research. γ-32P-ATP was received from Perkin-Elmer. All other reagents were obtained from Sigma or Mallinckrodt.
Synthesis of Platinated Oligonucleotides. Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer (Model 392) and purified by conventional methods. The platinated oligonucleotides 14GTG and 21GTG, in which the Pt atom forms 1,3-cross-link between two guanine bases, were prepared, purified and characterized as described previously.(16)

Preparation of 5'-End Radiolabeled Site-Specifically Platinum-Modified DNA Probes. The synthesis of DNA duplexes containing a unique, site-specifically platinum-modified DNA adduct was performed by enzymatic ligation of complementary oligonucleotides as previously described.(17, 18) In brief, an equimolar amount of 5'-32P-labeled 63-mer was combined with the site-specifically cisplatin-modified oligonucleotide and three other oligonucleotide fragments. All fragments were annealed and then incubated with T4 DNA ligase at 16 °C for 16 h. The resulting ligation products were separated by denaturing PAGE and subsequently isolated by non-denaturing PAGE. Unmodified DNA probes were synthesized and purified in the same manner as their platinated counterparts.

Nucleosome Reconstitution. The 5'-32P-radiolabeled DNA probes (146GTG, 146GTG-Pt, n146GTG and n146GTG-Pt) were assembled into nucleosomes in a manner similar to that in a previous report, except the glycerol was excluded from the nucleosome assembly buffer.(16) The nucleosomes were used as substrates for footprinting assays. The 5'-32P-labeled 199GTG-Pt or 199GTG mononucleosomes were assembled into nucleosomes and purified in the same manner as described.(16)
**Restriction Enzyme Digestion.** About 1 fmol of 5'-\(^{32}\)P-labeled 199GTG-Pt or 199GTG mononucleosomes or the corresponding free DNAs were treated with a restriction enzyme (20 U EcoRI, 5 U BsaAI, or 8 U PflM I) at 37 °C for 60 min. The reaction products were then treated with proteinase K and SDS at 30 °C for 15 min, and then phenol extracted and ethanol precipitated. The reactions were analyzed on an 8% denaturing PAGE gel.

**DNase I Footprinting Assay.** About 10 fmol of 5'-\(^{32}\)P-labeled 146merGTG or 146GTG-Pt mononucleosome or the corresponding the free DNA was treated with 0.2 U DNase I at 25 °C for 1 min or 5 min in TE buffer (10 mM Tris HCl (pH = 8.0), 1 mM EDTA). The reactions were quenched by the treatment with proteinase K and SDS at 30 °C for 15 min, followed by phenol extraction and ethanol precipitation steps. The reaction products were analyzed on a 6% denaturing PAGE gel.

**Hydroxyl Radical Footprinting Assay.** A hydroxyl radical footprinting assay was carried out according to published methods(19, 20) with reconstituted nucleosome substrates but omitting the step involving sucrose gradient centrifugation. About 10 fmol of 5'-\(^{32}\)P labeled mononucleosome or free DNA were treated with 0.6% H\(_2\)O\(_2\), 1 mM Fe(II)-2 mM EDTA, and 2 mM sodium ascorbate. Reactions were quenched with 5% glycerol. The mixtures were loaded onto a 5% native PAGE gel to separate the nucleosome and free DNA bands. The labeled DNA substrates were excised, purified separately and analyzed on a 6% denaturing PAGE gel.
Results and Discussion

Synthesis of 5'-End Radiolabeled Site-Specifically Platinum-Modified DNA Probes.

To test the effect of cisplatin modification on nucleosome structure, two similar site-specifically platinum-modified DNA probes and their unmodified counterparts were synthesized by enzymatic ligation (for sequences see Figures 3.1 and 4.1). The site of cisplatin cross-link was shifted by about half a turn of a double helix toward the 3'-end in the n146GTG-Pt probe compared to the 146GTG-Pt substrate, in order to provide two different rotational configurations with respect to the nucleosome octamer core. All four DNA probes were assembled into nucleosomes and characterized by gel electrophoresis (Figure 4.2). Band corresponding to the nucleosomes were excised from the gel to provide material for the footprinting experiments.

Restriction Enzyme Assay. BsaA I, EcoR I and PflM I were used to probe DNA accessibility near the center of the nucleosome core particle and of the linker region of the 199 mer-containing nucleosome (sequence see Figure 2.1). The purified 5'-end radiolabeled site-specifically platinum-modified nucleosome and the unmodified nucleosome were prepared and analyzed on a 5% non-denaturing PAGE gel (Figure 4.3). The cut bands were quantified and are indicated in Figure 4.4. Both unmodified and modified nucleosomes are cleaved with significantly lower efficiency than their corresponding free DNAs. The platinum adducts on the free DNA did not affect the cleavage efficiency of any enzyme, whereas the platinated nucleosome has relatively low cleavage efficiency by PflM I and EcoR I, compared to the unmodified
nucleosome. This result indicates that the local distortion induced by the cisplatin damage changes the accessibility and recognition efficiency of the nuclease for DNA in the nucleosome. In particular, our data suggest that these sites are facing the histone core as a consequence directing the site of platinum cross-link toward the outside of the nucleosome particle (see below).

**DNase I Footprinting Characterization of the Nucleosomes.** The DNase I footprinting results for nucleosome substrates 146GTG and 146GTG-Pt revealed ~10 bp periodicity, whereas a greater number of bands were observed for the DNA substrates alone (data not shown).(21, 22) The cleavage rate of nucleosomal DNA is much slower than that of free DNA, indicating that the core histones protect the DNA from DNase I digestion. Comparison of the cleavage pattern from platinated versus unmodified nucleosomes revealed differences over the region of 30 bp to 120 bp (see Figure 4.5).

**Characterization of Nucleosomes by Hydroxyl Radical Footprinting.** In contrast to DNase I, the small size of hydroxyl radicals makes them relatively non-specific cleavage agents. The hydroxyl radical footprinting method has been used to map the location of DNA-binding drugs, such as distamycin, actinomycin, netropsin, and mithramycin.(23-26) Hydroxyl radical footprinting was applied here to examine the rotational settings of the nucleosomal DNAs 146GTG-Pt and 146GTG by measuring the accessibility of the minor groove and DNA phosphate backbone to the solvent.(19) The cleavage pattern of nucleosomal DNA exhibited ~10 bp periodicity, a characteristic feature of the nucleosome structure,(2, 27) which is absent in naked
DNA. Cisplatin-modification does not disrupt the overall nucleosome structure, since the 10 bp pattern is also observed in modified nucleosome. More strikingly, the 146GTG-Pt nucleosome exhibits three strong bands corresponding to the site of GTG platinum cross-link (Figure 4.6). This result indicates the DNA backbone of the cisplatin damage site faces outward and is more exposed to hydroxyl radicals than other positions in the modified nucleosome. Another characteristic feature is that the peaks in the cleavage pattern are shifted about 2-3 bp towards to 3'-end of the 146GTG-Pt nucleosome for two to three helical turns near the cisplatin-DNA adduct compared to the cleavage pattern of the unmodified nucleosome. This shift indicates that the change in rotational setting most likely propagates to two or three adjacent turns. In addition, the most dramatic change in the cleavage pattern occurs \pm 1 helical turns around the damage site indicating that the cisplatin-DNA adduct induces a significant local distortion.

To check the effect of the cisplatin-DNA d(GpTpG) cross-link on rotational position, we synthesized a new platinated probe n146GTG-Pt, in which the platinum atom was placed half of a helical turn towards to the 3'-end of the 146mer; its unmodified counterpart n146GTG was also prepared.

A nucleosome model was built up based on the solved nucleosome crystal structure on a similar DNA sequence,(2) using MOLMOL software. As shown in Figure 4.7, if incorporation of the cisplatin-cross-link did not alter the rotational setting of the DNA, the sugar-phosphate backbone in the vicinity of the cisplatin-DNA adduct should faced toward the inside of the nucleosome and be shielded by
the histone core. However, three strong bands corresponding to the GTG platinum damage site occur in the hydroxyl radical footprinting from this new probe (Figure 4.8). As for the previous probe, the cleavage pattern peaks also shift by 1-2 bp over two-three helical turns surrounding the site of the cisplatin-DNA adduct. Thus, formation of cisplatin-DNA adducts changes the rotational setting by about 180°.

That DNA lesions affect the rotational setting of nucleosomal DNA seems to be a general phenomenon. Other DNA lesions, such as UV photo-products, also affect nucleosome rotational setting,(28, 29) as does the bisintercalating antitumor antibiotic echinomycin and several non-intercalating groove binding ligands. DNA rotation by 180° on the protein core are not unprecedented.(30-33)

The rotational setting of DNA in the nucleosome is determined by the anisotropic flexibility of DNA and the helical periodicity of defined structural properties.(34, 35) The resulting bending in a preferred direction determines which sequences will face in or out, relative to the histone octamer.(36) Therefore, the rotational settings surrounding a DNA lesion must be the consequence of the combined forces of reduced DNA flexibility and the DNA bending by both the nucleosome and the lesion. Changes of the rotational setting may be necessary in order to lower the total energy of modified nucleosome. Some regulatory factors can bind to nucleosomal DNA only if the target sequence is presented in a particular rotational orientation on the histone surface.(37) It is conceivable that the accessibility of DNA damage is modulated by changing the rotational setting for the specific purpose of attracting transcription factors or other DNA-binding proteins.
The formation of DNA lesions modulates the stability of nucleosomes containing the damaged DNA. Benzo[a]pyrene diol epoxide (BPDE) adducts significantly enhance nucleosome formation, whereas UV photoproducts inhibit the process for the same sequence. The effects of cisplatin may depend on both location and sequence. We observed less stable nucleosomes for 146GTG-Pt than for the unmodified 146GTG nucleosome (shorter storage time at 4 °C). However, we observed the nucleosome containing the cisplatin-modified DNA n146GTG-Pt is more stable than that of n146GTG (data not shown). The detailed mechanisms by which cisplatin-adducts alter nucleosome formation remain to be elucidated.

In conclusion, we have characterized site-specifically cisplatin-modified nucleosomes and DNA by restriction enzyme and DNase I digestion analysis, along with hydroxyl radical footprinting. Cisplatin-DNA adducts change the rotational settings of, and enzyme accessibility to, the DNA. These changes may contribute to the biological properties of cisplatin.

Acknowledgment

I thank A. Danford for assistance in purifying some of the oligonucleotide fragments and some experiments. I thank Qun Wang and Dr. T. D. Tullius for the technical help of hydroxyl radical footprinting experiment. I also acknowledge members of our laboratories for helpful discussion and comments on the manuscript.
References


Figure 4.1. Strategy for synthesizing site-specifically platinated-DNA probe. Five synthetic oligonucleotides, one bearing a 1,3-d(GpTpG) intrastrand platinum-DNA cross-link, were annealed and ligated as shown. Sequences of the individual strands are depicted.
Figure 4.2. Non-denaturing PAGE gels of nucleosomal and free DNA. A. n146GTG (unmodified) and n146GTG-Pt (cisplatin-modified) are loaded onto a 5% non-denaturing PAGE gel. Lane 1, cisplatin-modified nucleosome of n146 GTG-Pt; lane 2, cisplatin-modified free DNA of n146GTG-Pt; lane 3, unmodified nucleosome of n146GTG; lane 4, unmodified free DNA of n146GTG. B. 146GTG (unmodified) and 146GTG-Pt (cisplatin-modified) are analyzed on a 5% non-denaturing PAGE gel. Lane 1, unmodified nucleosome of 146GTG; lane 2, cisplatin-modified nucleosome of 146GTG-Pt; lane 3, cisplatin-modified free DNA of 146GTG-Pt; lane 4, unmodified free DNA of 146GTG.
Figure 4.3. Non-denaturing gel of $5'$-$^{32}$P-labeled nucleosomal DNA of 199GTG (unmodified) and 199GTG-Pt (cisplatin-modified). Lane 1, cisplatin-modified nucleosome 199 GTG-Pt; lane 2, unmodified nucleosome 199 GTG; lane 3, unpurified nucleosome 199GTG.
Figure 4.4. Restriction enzyme digestion of nucleosomes 199GTG and 199GTG-Pt. A. BsaA I and PflM I digestions of 199GTG and 199GTG-Pt nucleosomes. B. EcoR I digestion of nucleosome 199GTG and 199GTG-Pt. C. Locations of BsaA I, PflM I, and EcoR I cutting sites.
Figure 4.5. DNase I footprinting of cisplatin-modified and unmodified nucleosomes.

Lane 1, 100-bp DNA ladder; lane 2, 10-bp DNA ladder; lane 3, nucleosome 146GTG; lane 4, cisplatin-modified nucleosome 146GTG-Pt.
Figure 4.6. Hydroxyl footprinting of the 146GTG and 146GTG-Pt nucleosomes. Both nucleosome substrates showed 10-bp periodicity in their cleavage pattern. The modification sites of 1,3-d(GpTpG) (70-72) are indicated. The lengths of bands are also indicated.
Figure 4.7. Model of the n146GTG nucleosome. The nucleosomal DNA is shown in gray and the histone core particle is omitted for the clarity. The platinum-bound guanines of 1,3-d(GpTpG) (76-78) are colored in green. The sugar-phosphate backbone of 1,3-d(GpTpG) is buried inside.
Figure 4.8. Hydroxyl footprinting of the n146GTG and n146GTG-Pt nucleosomes. Both nucleosome substrates showed 10-bp periodicity in their cleavage pattern. The modification sites of 1,3-d(GpTpG) (76-78) and the lengths of bands are indicated.
Chapter 5  Cisplatin-Induced Post-Translational Modification of Histones H3 and H4*

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Introduction

Cisplatin \([\text{cis-diamminedichloroplatinum(II)}]\),\(^1\) widely used to treat a variety of human cancers, binds to DNA to form covalent platinum-DNA adducts.\((1,2)\) Platination of the genome triggers cellular responses involving several pathways, including DNA repair, transcription inhibition, cell cycle arrest and apoptosis, all of which require remodeling of the structural and dynamic properties of chromatin.\((3-6)\)

The p38 mitogen-activated protein kinase (MAPK) pathway has been implicated in osmotic stress, cell cycle regulation, differentiation, inflammation, development and many other biological processes.\((7-12)\) In particular, the p38 MAPK pathway is involved in the cellular response to various DNA damaging agents, including UV irradiation and cisplatin.\((13-15)\) Recently, a growing body of evidence suggests that activation of the p38 MAPK pathway plays an important role in the therapeutiic response to cisplatin.\((16,17)\) Cisplatin, but not the inactive stereoisomer \trans-DDP\ and not the \Pt(IV) compound PtCl\(_4\), induces long-term activation of p38 MAPK.\((16)\) This kinase regulates immediate-early (IE) gene expression and other cellular responses by phosphorylating various substrates, including transcription factors, chromatin proteins, and downstream Ser/Thr effector kinases.\((18)\) Understanding the detailed mechanism of signal transduction following of cisplatin-stimulated cellular response could lead to the rational design of better drugs and more efficient therapy.

\(^1\)The abbreviations used are: cisplatin, cis-diamminedichloroplatinum(II); MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; PBS, phosphate-buffered saline; IE gene, immediate-early gene; HAT, histone acetyltransferase; HDAC, histone deacetylases; JNK, \(c\)-Jun NH\(_2\)-terminal kinase; ERK, extracellular signal-regulated kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Histone post-translational modification regulates chromatin structure and cell division.(19-23) Phosphorylation of histone H3 at Ser 10 has dual, and opposing, roles in interphase and metaphase. During interphase, phosphorylation of H3 at Ser 10 can facilitate transcription of the IE genes,(24-28) whereas during mitosis, such phosphorylation facilitates chromosome remodeling and condensation.(29-31) Several stimuli including UV irradiation, arsenite treatment, and EGF (epidermal growth factor) bring about phosphorylation of histone H3, but until now, it was unknown whether cisplatin could induce such phosphorylation. In present study, we investigated the phosphorylation state of histone H3 in cells treated with cisplatin as well as the signal transduction pathway mediating such an event. We discovered that the drug triggers specific phosphorylation of histone H3, mediated by p38 MAPK pathway. We further report that hyperacetylation of histone H4 also occurs. These findings reveal a direct link between cisplatin treatment and chromosome modifications that can lead to structural changes with attendant modulation of function.

METHODS AND MATERIALS

Reagents. Cisplatin was obtained as a gift from Johnson-Matthey. Antibodies were purchased from Upstate Biotechnology. Sodium butyrate was purchased from Sigma. DMEM, RPMI-1640 medium and L-glutamine were obtained from Invitrogen. SKF86002 was bought from Calbiochem.
Cell Culture. HeLa and MCF-7 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum on tissue culture plates at 37 °C in a humidified atmosphere of 5% CO₂. Ntera II cells were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum and 2 mM glutamine on tissue culture plates at 37 °C in a humidified atmosphere of 5% CO₂. Cells were grown in medium to near 90% confluence, then pretreated with various concentrations of cisplatin for fixed times before the preparation of nuclear extracts. For studies with the p38 MAPK inhibitor SKF86002, cells were pre-incubated with 10 μM of the inhibitor for 1 hr before cisplatin treatment.

Preparation of Nuclear Extracts. Nuclear extracts were prepared from cells as described in the Clontech TransFactor Extraction Kit (PT3612-2) with a slight modification. Briefly, HeLa cells were washed twice with 5 packed cell volumes of cold phosphate-buffered saline (PBS). The cells were collected and transferred to clean centrifuge tubes, then centrifuged for 5 min at 450 X g, after which the pellets were rinsed twice with an equal volume cold PBS. The cell pellets were resuspended with 5 packed cell volumes of ice-cold lysis buffer (10 mM Hepes (pH = 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 2 mM PMSF) and incubated on ice for 15 min. The cells were spun down and the pellets were resuspended with 2 packed cell volumes of ice-cold lysis buffer. The cells were lysed with 10 strokes of a narrow-gauge syringe (No. 263/4). The suspension was spun at 11,000 X g for 20 min. The pellet was resuspended in a two-thirds volume extraction buffer comprising 20 mM Hepes (pH = 7.9), 25% glycerol, 0.42 M NaCl, 1 mM DTT, 2 mM PMSF, and 1.5 mM MgCl₂. The solution was homogenized
with the narrow-gauge syringe. The suspension was shaken for 30 min at 4 °C. The nuclear extract was spun at 20,000 X g for 5 min at 4 °C. The supernatant was transferred in small aliquots and stored at -80 °C.

**Immunoblotting.** Proteins were analyzed on a 4-20% SDS-PAGE minigel (Bio-Rad). Membranes containing proteins were incubated with specific histone antibodies as indicated in the figures and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody separately, and were detected with the chemiluminescence ECL plus detection system (Amersham Pharmacia Biotech). Actin was used as the loading control.

**RESULTS**

*Cisplatin Strongly Induces Histone H3 Ser 10 Phosphorylation in HeLa Cells.* To study whether cisplatin activates a pathway leading to phosphorylation of histone H3 at Ser 10, HeLa cells were pretreated with 1 µM of the drug for 16 h. HeLa nuclear extracts were prepared and analyzed by western blotting using a phospho-specific antibody against phosphorylated Ser 10 of histone H3. Cisplatin strongly induces the phosphorylation of histone H3 at Ser 10 (Figure 5.1A). A time course study revealed that platinum-stimulated phosphorylation of histone H3 at Ser 10 increases monotonically with incubation time from 1 to 24 hr (Figure 5.1B). The phosphorylation level of histone H3 at Ser 10 also rises with increasing concentrations of cisplatin (Figure 5.1C). *trans*-DDP, a stereoisomer of cisplatin that is not active against cancer, has no significant effect on the degree of H3 Ser 10 phosphorylation compared with cisplatin under the same experimental conditions (Figure 5.2, lane 1).
Cisplatin Induces Histone H3 Phosphorylation in Other Cell Lines. To address whether this cisplatin-induced phosphorylation of histone H3 at Ser 10 is cell specific, we also tested MCF-7 breast cancer cells and the Ntera II testicular cancer cells. Cisplatin induces phosphorylation of histone H3 at Ser 10 in both of these cell lines. Interestingly, the induction of histone H3 phosphorylation was significantly greater in the Ntera II than the MCF-7 cells (Figure 5.3).

SKF86002 Inhibits Phosphorylation of H3 at Ser 10 Activated by Cisplatin. Cisplatin causes activation of p38 MAP kinase. To explore whether p38 MAPK has a role in mediating cisplatin-induced phosphorylation of histone H3, we tested the effect of a specific p38 MAPK inhibitor, SKF 86002, on the cisplatin-induced phosphorylation of histone H3 Ser 10. We prepared nuclear extracts from HeLa cells pretreated or untreated with SKF86002. The western blot results revealed that the phosphorylation level of H3 Ser 10 induced by cisplatin is significantly reduced when cells were pretreated with SKF86002 (Figure 5.2). This result suggests that cisplatin-induced phosphorylation of histone H3 at Ser 10 is mediated by the p38 MAPK pathway.

Cisplatin Induces Other Histone Modifications. To explore other possible histone modification pathways activated by cisplatin, we analyzed HeLa nuclear extracts by western blot using several modification-specific antibodies. The results demonstrate that cisplatin also induces phosphorylation of H3 at Ser 28 (Figure 5.4A) and acetylation of histone H4 (Figure 5.4B).
DISCUSSION

In the present study, we discovered that cisplatin treatment strongly induces phosphorylation of histone H3 at Ser 10 in HeLa cells. Phosphorylation of this amino acid is associated with mitotic and meiotic chromosome condensation,\(^{(22,29,31)}\) helps to regulate transcription,\(^{(24)}\) and is brought about by various stimuli, including TPA (12-O-tetradecanoylphorbol-13-acetate), EGF, UV irradiation and arsenite.\(^{(27,32,33)}\)

Cisplatin, a potent anti-cancer drug, forms platinum-DNA adducts and causes a variety of cellular responses. However, a relationship between cisplatin treatment and histone H3 modification had not previously been established.

Cisplatin activates p38 MAPK for 8-12 h in sensitive cells and transiently for 1-3 h in resistant cells.\(^{(16,17)}\) Moreover, lack of p38 MAPK activation/function induces a resistant phenotype in human cells.\(^{(16,17)}\) MAP kinases may also play a role in modifying the chromatin environment of target genes. Here we show that SKF86002, a potent p38 inhibitor, inhibits cisplatin-induced phosphorylation of histone H3 at Ser 10. Furthermore, the p38 MAP kinase and downstream kinase MSK-1 have been reported to phosphorylate histone H3 Ser in vitro.\(^{(13,27)}\) Taken together, this evidence indicates that p38 MAPK mediates phosphorylation of histone H3 at Ser 10 in response to cisplatin.

Our results also indicate that the inactive isomer of cisplatin, trans-DDP, fails to induce phosphorylation of histone H3 at Ser 10. Previous work revealed that both cisplatin and trans-DDP activate p38 MAPK differently. Only the active drug cisplatin
can bring about long-term activation. This difference in the kinetics of activation could account for their differential cytotoxicity.

Cisplatin also modestly induces phosphorylation of histone H3 at Ser 28. Phosphorylation at this site can be also triggered by UV irradiation, TSA and other stimuli. The kinetics are very similar to those for Ser 10. Both Ser 10 and Ser 28 on histone H3 N-terminal tails are highly conserved and occur in the same consensus sequence, ARKS.

There are two distinct modes of histone H3 phosphorylation, mitotic and stimulus-inducible. Both Ser 10 and Ser 28 on histone H3 are highly phosphorylated on condensed chromosomes during mitosis. The exact role of histone phosphorylation is still not fully understood, although several models have been proposed to explain such a process during mitosis. Diverse stimuli induce rapid phosphorylation of H3 at Ser 10, which has been correlated with transcription activation of certain IE genes, by changing the nucleosome environment or serving as a binding motif for recruiting co-activators or chromatin remodeling complexes.

Cisplatin-induced phosphorylation of histone H3 also occurs in other cell types than HeLa. In testicular cancer cell lines, a high level of H3 phosphorylation is observed. Since the Ntera II cell line is at least twice as sensitive to cisplatin than MCF-7 cells (data not shown), there may be a link between H3 phosphorylation and cisplatin cytotoxicity.

Acetylation of histones correlates well with transcription activation of many genes, presumably by increasing the accessibility of particular genomic regions for transcription activator or remodeling protein complexes. The acetylation of histones is
under the control of histone acetyltransferases (HATs) and histone deacetylases (HDACs). We find that cisplatin treatment induces hyperacetylation of histone H4. Although this process could occur by a mechanism independent from that by which histone H3 is phosphorylated, it might be that MAP kinase-dependent recruitment of co-activators with HAT activity to sequence-specific regulatory elements contributes to acetylation of histones, as has been reported. Acetylation and phosphorylation of histones are linked with transcriptional activation and facilitate DNA repair.

Phosphorylation of histone H3 Ser 10 is also induced by UV, arsenite, EGF and other stimuli through activation of the MAPK pathway (Table 1). In general, the extracellular signal-regulated kinases (ERKs) are activated by mitogenic and proliferative stimuli, such as EGF and TPA. The c-Jun NH2-terminal kinases (JNKs) and p38 MAPKs respond to environmental stress and chemotherapeutic drugs, such as UV, arsenite, and cisplatin. The different patterns of MAPK pathway activation caused by these various stimuli may represent a regulatory signal by which cells respond to stress in a stimuli-specific manner.

Besides p38 MAPK, both JNK and ERK1/2 have been implicated in the cellular response to cisplatin. ERK was weakly activated (2-3 fold) by a 33 μM cisplatin treatment for 24 h, whereas JNK was more significantly activated (10-fold). Another study showed no activation of ERK 1/2 in HaCaT cells following a 33-132 μM cisplatin treatment for 4 h, whereas JNK and p38 MAPK could be activated by 33 μM cis-DDP. In addition, a third group reported no activation of ERK1/2 in HeLa cell lines cells after a 10 μM cisplatin treatment for 12 h, even though ERK activation was
observed at higher cisplatin concentrations. (43) Taken together, these results make it unlikely that ERK1/2 was activated by cisplatin under the experimental conditions (1 μM) whereby H3 was phosphorylated. Although we do not exclude other kinases for the cisplatin-induced phosphorylation of histone H3, the MAPK pathway seems to be a prominent one.

Cisplatin-DNA adducts arrest the cell cycle. (44-46) Nucleotide excision repair complexes are then recruited to repair the DNA damage. p38 MAPK-mediated phosphorylation and acetylation of histones causes nucleosomal structural changes, increasing accessibility of other proteins. These events facilitate the binding of the remodeling complex and repair proteins to the nucleosome, thereby stimulating DNA repair (39,40,47) and helping cells to survive cisplatin-induced stress. When DNA damage is extensive, cells will undergo apoptosis. Phosphorylation and acetylation of histones leading to the activation and transcription of IE genes will facilitate apoptosis under these conditions.

ACKNOWLEDGMENT

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REFERENCES


Table 5.1. Stimuli that Activate a MAPK Pathway to Induce Histone Phosphorylation

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<tr>
<td>TPA</td>
<td>ERK</td>
<td>H3 Ser 10/28 phosphorylation (25)</td>
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Figure 5.1. Cisplatin-induced phosphorylation of histone H3 at Ser 10. HeLa nuclear extracts were analyzed by western blot using an antibody against phospho-Ser 10 of histone H3. A, lane 1: HeLa nuclear extract (control); lane 2: HeLa nuclear extract pretreated with 1 μM cisplatin overnight. B, time-course of cisplatin-induced phosphorylation of histone H3 Ser 10. HeLa nuclear extracts were prepared from cells pretreated with 10 μM cisplatin for 0, 1, 4, 8, 12, or 24 h. C, dose-dependence of cisplatin-induction of phosphorylation of histone H3 Ser 10. HeLa cells were treated with 0, 0.2, 1.0, 10, or 40 μM cis-DDP for 8 hr before harvesting.

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Figure 5.2. Inhibition of cisplatin-induced phosphorylation of H3 Ser 10 by SKF86002. HeLa nuclear extracts were pretreated with either by 1 μM trans-DDP (lane 1); 1 μM cisplatin and 10 μM p38 MAPK inhibitor (SKF86002) (lane 2); 10 μM p38 MAPK inhibitor (SKF86002) (lane 3); or 1 μM cisplatin (lane 4) overnight. Lane 5 shows a HeLa nuclear extract control. HeLa nuclear extracts were analyzed as described in the caption to Fig. 5.1.
Figure 5.3. Cisplatin induction of phosphorylation of histone H3 in MCF-7 (top) and Ntera II (bottom) cell lines. Lane 1: nuclear extracts (control). Lane 2: nuclear extracts prepared from cells pretreated with 1 μM cisplatin overnight. Nuclear extracts were analyzed as described in the caption to Fig. 5.1.
Figure 5.4. Cisplatin induction of phosphorylation of H3 Ser28 and acetylation of H4. A, HeLa nuclear extracts were analyzed by western blot using antibody against phospho-Ser 28 of histone H3. Lane 1: HeLa nuclear extract (control). Lane 2: HeLa nuclear extract pretreated with 1 μM cisplatin overnight. B, nuclear extracts prepared from HeLa cells were analyzed by western blot using anti-acetyl-lysine antibody or anti-acetylated histone H4 as indicated. Lane 1: HeLa nuclear extract (control). Lane 2: HeLa nuclear extract pretreated with 1 μM cisplatin overnight. Lane 3: HeLa nuclear extract pretreated overnight with 4 mM sodium butyrate, an inhibitor of histone deacetylases (positive control).
Chapter 6  The Modulating Effect of DNA Methylation on the Cellular Processing of Cisplatin-Damaged DNA
Introduction

Cisplatin, cis-diamminedichloroplatinum(II), has been used to treat various cancers for over 25 years. The drug interacts covalently with DNA forming mostly 1,2- and 1,3-intrastrand cross-links. These platinum-DNA adducts trigger several cellular responses, including transcription inhibition, DNA repair, cell cycle arrest, and apoptosis.(1) Understanding the detailed mechanisms by which cells process such cisplatin damage, identifying the factors that manipulate the processing and unraveling the cytotoxic mechanism of the drug hold promise of providing rationales to improve the efficacy of the drug and to develop new anticancer agents.

Epigenomic regulation of genes is essential for proper cellular function. One major form of epigenetic information in mammalian cells is DNA methylation, mostly 5-methylcytosine within a d(pCpG) dinucleotide. DNA methylation has profound effects on the mammalian genome, with approximately 3–5% of the cytosine residues in genomic DNA existing as 5-methylcytosine.(2) Hypermethylation of the promoter region of a gene silences its expression. In addition, DNA methylation also modulates chromatin structure, inactivates the X chromosome, and affects DNA repair and genomic imprinting.(3–8) The pattern of genomic methylation is conserved even following DNA replication.(9)
Cancer cells frequently exhibit altered genomic methylation patterns, mostly showing global hypomethylation with region-specific hypermethylation within the promoter region of certain genes. These aberrant patterns of DNA methylation have been linked to the onset of cancer presumably by silencing the expression of tumor suppressor genes. It is therefore of interest to investigate how such different methylation profiles might affect the processing of cisplatin-DNA lesions in cancer and normal cells.

In the present study we have examined the role of DNA methylation in the formation of platinum-DNA adducts, on protein-DNA interactions, and on the repair of platinum damage. The rates and extents of platination of DNA probes containing 5-methylcytosine residues adjacent to the platinum binding site were compared to those for non-methylated DNA. The effect of DNA methylation on the interaction of a protein with platinated DNA was studied for domA of HMGB1 binding to methylated and non-methylated DNA probes. Finally, the effect of DNA-methylation on the NER of cisplatin-modified DNA was investigated using 195mer substrates containing site-specifically cisplatin-modified DNA with adjacent 5-methylcytosine residues.

**Materials and Methods**

*Materials.* Cisplatin was obtained as a gift from Engelhard. Reagents for DNA synthesis were purchased from Glen Research. SequaGel diluent and concentrate solutions for preparing denaturing polyacrylamide gels were
purchased from National Diagnostics. Hydrazine (98%, anhydrous), dimethyl sulfate, and other reagents for DNA sequencing were purchased from Aldrich. T4 polynucleotide kinase, T4 DNA ligase, and NEB buffer 3 were obtained from New England Biolabs. γ-32P-ATP was purchased from PerkinElmer. The analytical and preparative ion exchange high-performance liquid chromatography (HPLC) columns (Dionex DNApac PA100) and analytical C-18 reverse phase HPLC columns were obtained from Dionex.

**Synthesis of Oligonucleotide Fragments.** All oligonucleotides (sequences are supplied in Appendix 6.1) were synthesized by using an Applied Biosystems DNA Synthesizer (Model 392) and purified by denaturing polyacrylamide gel electrophoresis. The gel slices containing the desired oligonucleotides were excised and eluted with 300 μL of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS).

**Preparative-Scale Platination of 16mer Fragments.** Four different single-stranded platinated 16mer fragments (16mer GG-Pt, 16mer GTG-Pt, 16mer GGMe-Pt, and 16mer GTGMe-Pt) were prepared in the same manner as described (13). Briefly, a portion of 16mer (100 nmol) was incubated in the dark with one equivalent of cisplatin in 0.01 M sodium phosphate buffer (pH = 6.75) at 37 °C for 18 h. Platinated oligonucleotides were then purified by ion exchange HPLC utilizing the gradient in Table 6.1. Following HPLC purification, the 16mer samples were analyzed by mass spectrometry and UV-vis (Hewlett-Packard 8453) and AA spectroscopy (Perkin-Elmer HGA 800).
Time Course of the Platination of Double-Stranded 14mer and Single-Stranded 16mer Substrates. The time-course for the formation of Pt adducts on four different single-stranded 16mer fragments was investigated. An aliquot of oligonucleotide (20 nmol) was incubated in the dark with 20 nmol of cisplatin in a total volume of 180 µL containing 0.01 M sodium phosphate (pH = 6.75) at 37 °C. A 10 µL aliquot was removed from the reaction mixture after 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. The extent of platination at different time points was evaluated by ion exchange HPLC utilizing the gradient in Table 6.2. The area under the peak for the platinated product was compared with that for the peak corresponding to the starting material. In a similar manner, 20 nmol of 14mer duplex DNA was incubated with 20 nmol of cisplatin in a total volume of 180 µL of 0.01 M sodium phosphate (pH = 6.75) at 37 °C. A 10 µL aliquot was removed from the reaction mixture after 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. The extent of platination was evaluated by reverse phase HPLC using a C-18 analytical column and utilizing the gradient in Table 6.3. The area under the peak corresponding to platinated DNA product was compared with that of the starting material peak.

Maxam-Gilbert Sequencing of 16mer and 36mer DNAs. The 16mer and 36mer fragments were sequenced to verify the presence and location of the 5-methylcytosine residues as well as to characterize the platinum-DNA crosslink. Maxam-Gilbert reactions were performed as described.(14)
Briefly, the guanine-specific reaction (G-reaction) was conducted by adding 4 μL of 1 g/L salmon sperm DNA and 5 μL of 5'-32P-labeled DNA substrate (approximately 10,000 cpm) to 190 μL of dimethyl sulfate buffer (50 mM sodium cacodylate, 1 mM EDTA). A portion of 5 μL of 10% dimethyl sulfate was added to the solution, and the reaction was then incubated for 5 min at ambient temperature. The reaction was stopped by adding 50 μL of dimethyl sulfate stop solution (1.5 M sodium acetate, 1 M β-mercaptoethanol, 250 μg/mL yeast tRNA).

The purine-specific reaction (G + A reaction) was performed by incubating with a mixture of 4 μL of 1 g/L salmon sperm DNA, 10 μL of 5'-32P-labeled DNA substrate (approximately 20,000 cpm), 10 μL of doubly distilled water, and 4 μL of 1 M piperidine formate (pH 2.0) at 37 °C for 15 min. The reaction was stopped by adding 240 μL of stop solution A (0.3 M sodium acetate, 0.1 mM EDTA, 100 μg/mL yeast tRNA).

The pyrimidine-specific reaction (C + T reaction) was performed by combining 4 μL of 1 g/L salmon sperm DNA, 10 μL of 5'-32P-labeled DNA substrate (approximately 20,000 cpm), 10 μL of doubly distilled water, and 30 μL of hydrazine (anhydrous, 98%). The reaction was incubated at ambient temperature for 7 min. The reaction was then stopped by adding 200 μL of stop solution A.

The cytosine-specific reaction (C-reaction) was conducted by combining 15 μL of 5 M sodium chloride, 4 μL of 1 g/L salmon sperm DNA, 10 μL of 5'-32P-
labeled DNA substrate (approximately 20,000 cpm), and 30 μL of hydrazine (anhydrous, 98%). The reaction was first incubated at ambient temperature for 5 min, and was then stopped by adding 200 μL of stop solution A.

For each of the sequencing reactions, the DNA was cleaved by piperidine. Briefly, the DNA was precipitated from solution with ethanol and then incubated in 100 μL of freshly prepared 1 M piperidine in water at 90 °C for 30 min. The piperidine was removed under vacuum. Substrates were treated with 50 μL of 0.2 M sodium cyanide overnight at 37 °C in order to remove the platinum from the DNA. Following the piperidine treatment, the samples were then analyzed by denaturing polyacrylamide gel electrophoresis. The intensity of the bands in each lane was quantified by ImageQuant Software (Molecular Dynamics).

Synthesis of Site-Specifically Platinated 195mer Substrates. The 195mer probes for the repair assay were synthesized from six oligonucleotides in the same manner as described.(13) Briefly, the substrates were constructed as indicated in Figure 6.1. For each 195mer, the 36mer, 72mer, and 96mer oligonucleotides were phosphorylated by using T4 polynucleotide kinase and ATP. The 16mer fragments were 5′-32P-labeled with T4 polynucleotide kinase and γ-32P-ATP. All the oligonucleotides were annealed and then incubated with T4 DNA ligase at 16 °C for 16 h. The 195mer products were isolated by denaturing PAGE, and eluted from the gel in elution buffer. The products from the denaturing gel were then re-annealed and further purified on a non-denaturing PAGE electrophoresis.
**Nucleotide Excision Repair Assay of 195mer DNAs.** The platinated methylated or non-methylated 195mers were used as substrates for the nucleotide excision repair assay as described.(13) The AA8 CHO cell-free extracts were added into the mixture and incubated at 30 °C for 2 h. The reaction products were then treated with proteinase K (15.3 mg/mL) and SDS (10% W/V) at 30 °C for 15 min, followed by phenol extraction and ethanol precipitation. The repair products were analyzed on an 8% denaturing PAGE gel.

**Gel Electrophoresis Mobility Shift Assay of HMGB1 DomA with ds-16mers.** Gel electrophoresis mobility shift assays (EMSAs) of HMGB1 binding to either methylated or non-methylated oligonucleotide duplexes were performed in a similar manner as described elsewhere.(15) Typically, a portion of either methylated or unmethylated dsDNA probes (0.4-5 nM, 5000 cpm) were incubated with increasing amounts of HMGB1 dom A in 10 µl EMSA buffer (10 mM HEPES, pH = 7.5, 10 mM MgCl₂, 50 mM LiCl, 100 mM NaCl, 1 mM spermidine, 0.2 mg/mL BSA, and 0.05% Nonidet P40) at 30 °C for 1 h. The samples were then analyzed on a 5% non-denaturing PAGE gel at 4 °C.

**Results**

**Synthesis and Purification of Platinated Oligonucleotides for 195mer Repair Substrates.** Platinated oligonucleotides fragments for the synthesis of the 195mer substrates were successfully prepared and purified, the yield varying from 65% to 85% yield depending upon substrate (Figure 6.2). The identity of the 16mer
substrates was confirmed by mass spectrometry from Biopolymers lab (Table 6.4). The mass difference between non-platinated and platinated substrates is consistent with the addition of a \( \text{Pt(NH}_3\text{)}_2^2+ \) group. In addition, the difference in mass between doubly-methylated and non-methylated substrates matches the predicted value for two methyl groups. UV/Vis-AA ratios of the platinated DNAs also indicate one platinum bound to each DNA molecule (Table 6.5).

**Sequencing of 16mer and 36mer Substrates.** In the cytosine-specific Maxam-Gilbert reaction, the cytosine ring is attacked by hydrazine; as a result, the base is cleaved and ribosylurea is formed. The DNA is further cleaved with piperidine through \( \beta \)-elimination of the phosphates group. The presence of a methyl group on the 5-position of the cytosine ring interferes with action of hydrazine.(14) The efficiency of cleavage at the 5-methylcytosine residue is thus much lower than cleavage at an unmodified cytosine. Indeed, the Maxam-Gilbert reactions for the 16mers and 36mers revealed a distinct difference between methylated and analogous non-methylated substrates (Figures 6.3-6.9). The bands corresponding to the cytosine residues were clearly observed in cytosine-specific reaction, whereas the corresponding bands of methylated cytosine residues were diminished. Together with the mass spectrometry data, these results confirmed presence and the positions of the 5-methylcytosine residues.

In addition, the Maxam-Gilbert reaction also revealed the position of the platination site. The N-7 position of guanine is methylated by dimethyl sulfate during the G-reaction, which thereby makes the base susceptible to removal from
the sugar ring by hydrolysis. The presence of a platinum adduct bound to the
N(7) position of guanine is expected to block the addition of a methyl group at
that site, suppressing the appearance of a guanine band in the Maxam-Gilbert
chemical degradation of DNA. Indeed, the intensity of the bands corresponding
to the guanine residues of 16mers where the platinum is situated decreased by
approximately two-fold when compared to the intensities of the corresponding
bands in the non-platinated substrates (Figure 6.10). Therefore, the Maxam-
Gilbert G-reaction results together with mass spectrometry and UV/Vis-AA
analysis confirmed the presence of the 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand
cisplatin-DNA cross-links.

Time Course of Platination of 14mer and 16mer Substrates. For both the
single-stranded 16mer substrates and the double-stranded 14mer probes, the
presence of 5-methylcytosine residues reduced the rate at which cisplatin formed
platinum-DNA intrastrand cross-links. The overall extent of platination after
24 h was significantly lower for methylated substrates compared with non-
methylated substrates (Tables 6. 6-7 and Figures 6.11-13).

EMSA Assay of dsDNA with HMGB1 Dom A. To investigate the effect of
DNA methylation on HMGB1 dom A binding to the major cisplatin-DNA
adducts, the $^{32}$P-labeled site-specifically platinated 16GG-Pt, methylated
platinated 16GGMe-Pt and 16GG-Pt//Me probes were prepared for EMSA
studies (Figure 6.14). The presence of the methylated cytosine residues adjacent
to platinated guanine base moderately reduced the affinity of HMGB1 dom A for
cisplatin-modified DNA (16GGMe-Pt), whereas the presence of methylated cytosine opposite to a platinated guanine (16GG-Pt//Me) significantly abolished the interaction between the DNA probe and HMGB1 dom A.

**Effect of Methylation on NER of 195mer Substrates.** Ten different 195mers containing either 1,2-d(GpG) or 1,3-d(GpTpG) intrastrand platinum-DNA cross-links were successfully synthesized and purified (Figure 6.1 and 6.15A). The methylcytosines were located either on the opposite strand of the platinated guanines or flanking the platinated guanines on the same strand. The 195mer probes were used as repair substrates to determine whether or not the presence of adjacent 5-methylcytosine residues would modulate the NER of platinum-DNA adducts (Figure 6.15 B). The relative repair efficiencies are reported in Figure 6.15 C. Although the repair efficiency of methylated DNA is similar to the efficiency of non-methylated DNA, some modest effects of DNA methylation occur. First, the effect of methylation depends on the location of methylcytosines and type of cisplatin lesions. For 1,2-d(GpG) intrastrand cross-link, repair is slightly stimulated when two methylcytosines are located opposite the platinated guanines (Figure 6.15, lane 7 vs lane 10), whereas no stimulation is observed when the methylcytosines are located in flanking positions of platinated guanines (Figure 6.15, lane 6 vs lane 10). For 1,3-d(GpTpG) intrastrand cross-link, no change or slight inhibition of repair is observed when two methylcytosines are located opposite the platinated guanines (Figure 6.15, lanes 2 vs lane 5), whereas moderate inhibition is observed when the methylcytosines are located
in positions flanking the platinated guanines (Figure 6.15, lane 1 vs lane 5). Inducing mono-methylcytosine in the strand opposite platinated guanines also modulates excision repair in a location-specific manner (Figure 6.15, lane 3 vs lane 4; lane 8 vs lane 9).

Discussion

_DNA Methylation Modulates the Formation of Platinum-DNA Adducts._ The effect of cytosine methylation on the structure of DNA had been studied through a variety of means including X-ray crystallography, NMR spectroscopy, gel electrophoresis, FT-IR spectroscopy, and molecular dynamics simulations. Although no gross conformational changes occur by introducing a 5-methyl group on the cytosine base, (16-20) methylation causes certain local perturbations. In particular, methyl group alters the backbone dynamics, (21-23) bending flexibility, (24) and curvature of the DNA. (25,26) Moreover, recent studies revealed that 5-methylation changes the thermodynamic (free enthalpies) and kinetic properties of the DNA backbone. Through methylation, the GpC step is stabilized in the BI conformation by about 1.1 kcal/mol, whereas the BI substate of CpG is destabilized by 0.8 kcal/mol, although still energetically favored. The BI and BII are two conformations defined by different $\epsilon(C_4'-C_3'-O_2'-P)$ and $\zeta(C_3'-O_3'-P-O_5')$ torsion angles. The barrier for conversion of BI to BII in Gp$^{5mC}$ is increased by 1.5 kcal/mol compared to the non-methylated step GpC, and the barrier for $^{5m}CpG$ is lowered by 0.8 kcal/mol compared to CpG. (27) In addition,
solid-state deuterium NMR analysis revealed a significant reduction in the phosphodiester backbone mobility at the site of the methylated cytosine.(21) Substitution of hydrogen with 5-methyl group in cytosine increases melting temperature.(28) The 5-methyl group of the cytosine base enhances of the base stacking ability and changes the sugar pucker and the glycosidic bond torsional angle.(19,29) One X-ray crystallographic study revealed that the methyl group forms weak C-H...O hydrogen bond with anionic oxygen atoms of the phosphate group, which may stabilize the interaction.(30) These factors may contribute to the changes of DNA structure of methylated DNA and account for differences in protein-DNA interaction.

The formation of intrastrand cisplatin-DNA cross-links causes the adjacent guanines bases to roll toward each other, bending the helical axis of the host duplex in the direction of the major groove resulting a flatter minor groove opposite the platinum adduct. Moreover, such adducts partially disrupt the stacking interactions between neighboring base-pairs at and adjacent to the platination site.(1) Therefore, it is not surprising that methylated DNA is less reactive to cisplatin compared with non-methylated DNA. Several factors, such as reduced flexibility, increased base stacking, significantly reduced phosphordiester backbone mobility of methylated DNA may contribute to the diminished rate and extent of platination for the methylated DNA compared to unmodified DNA in vitro.
DNA Methylation Modulates the Interaction between HMGB1 Dom A and Cisplatin-DNA Adducts. Cisplatin modifications distort the structure of the DNA duplex, bending it significantly toward the major groove and exposing a wide, shallow minor groove surface for protein binding. Several classes of proteins, such as high mobility group box proteins, repair proteins, and transcription factors, recognize 1,2-d(GpG) intrastrand cross-linked platinum-DNA adducts. It is therefore of interest to investigate the effects of cytosine methylation on the interaction between these proteins and platinated DNA. We have examined HMGB1 dom A as a model to investigate such behavior.

HMGB1, a well-documented protein that preferentially binds to 1,2-d(GpG) cisplatin-DNA adducts, has two tandem HMG box domains and an acidic C terminus. The structure of the complex of HMGB1 dom A with a ds16mer containing a site-specific 1,2-d(GpG) cisplatin-DNA adduct has been described. A striking feature is the intercalation of the Phe 37 side chain into a hydrophobic notch in the minor groove formed by the cisplatin-DNA adduct. This intercalation is important for DNA affinity, since domA F37A mutant protein has extremely low binding affinity for the platinated DNA probe.

Previous studies revealed that alteration of the nucleotides flanking the platinum lesion modulated HMG-domain protein recognition of cisplatin-modified DNA. Here we demonstrate that the presence of methyl groups on cytosines flanking the platinum lesion also modulate HMG-domain protein affinity in cisplatin modified DNA. Moreover, when the 5-methylcytosines are
located in the strand opposite that containing the cisplatin-DNA adduct, the binding affinity is almost completely abolished. Such a striking effect is consistent with photo-crosslinking results that indicate significantly less photo-crosslinking of HMGB1 with platinated DNA containing methylcytosines in the opposite strand. (32) Moreover, the effect of adding an exocyclic methyl group also is consistent with results from a previous study, which revealed that HMGD binding affinity is stimulated upon a substitution of dT by dU. (33)

The inhibitory effect on HMGB1 binding caused by the exocyclic methyl group of cytosine has two possible origins. The presence of a methyl group near a cisplatin-DNA cross-links alter the local structure and thereby reduce the affinity for HMGB1 dom A. In addition, the methyl group may directly block a specific interaction of HMGB1 dom A with platinated DNA. The binding surface of the HMGB1 A domain extends exclusively to 3' side of the bend locaus caused by platination and covers five base-pair steps. Phe 37 intercalation plays a determining role in this protein-DNA interaction. It has been proposed that hydrophobic residues are employed as an interactive wedge to pry open one or several base pair steps, thereby bending the DNA away from the protein toward the major groove. (31,34) Methylated cytosines cause a local deformation of the major groove of DNA, inducing rearrangement of the methylated base pairs, which are then pushed into the minor groove causing a deeper major groove. (18,35) Therefore, the presence of methylcytosine may disrupt the intercalation of Phe 37 and serve as a stereochemical barrier to the compression of the major
groove required by the bending induced by HMGB1 domA. Moreover, helix I of HMGB1 dom A interacts with sugar-phosphate backbone of the bottom strand, the strand opposite to platinum cross-link, whereas helix II mainly contacts the backbone of the unmodified strand.(31) Since methylation changes the thermodynamic and kinetic properties of the DNA backbone, reducing the DNA flexibility by increasing stacking interactions, it may indirectly affect this interaction. An X-ray crystal structure of cisplatin-modified methylated DNA in complex with HMGB1 dom A would provide more detailed information.

The effect of DNA methylation on the binding affinity of HMGB1 for cisplatin-DNA adducts may have significant biological consequences. In vitro nucleotide excision repair studies indicate that HMGB1 shields the 1,2-d(GpG) intrastrand platinum cross-link from NER by stably binding to this DNA adduct.(36,37) Moreover, HMGB1 interacts with the nucleosome at the entry and exit points of the DNA, thus decreasing the compactness of the chromatin fibers by weakening the binding of H1 to nucleosomes. HMGB1 also facilitates binding of the remodeling complex ACF to nucleosomal DNA, thereby accelerating its ability to induce structural changes in the nucleosome.(38-40) Addition or deletion of the exocyclic methyl group might add another layer to modulate the affinity of this protein to DNA and thereby its function in vivo.

**DNA Methylation Modulates NER of Cisplatin-DNA Adducts.** The EMSA results reveal significant reduction in the binding affinity of HMGB1 dom A to methylated DNA probes. Since HMGB1 shields cisplatin-DNA lesions from
repair, one would expect that NER might be less inhibited for methylated DNA probes. However, we only observed a modest stimulation in NER for DNA methylated on the strand opposite platinated guanine residues. One explanation is that proteins binding to methylated DNA, such as methyl-CpG-binding domain (MBD) family proteins, shield the cisplatin-DNA adducts, like HMGB1.(41,42) MBD4 has been related to the cellular response to DNA damage and to affect DNA repair.(8,43-45) A specific role for MBD on NER of cisplatin-DNA damage remains to be determined.

The effects of DNA methylation on NER depend upon the lesion type and the specific flanking sequence and range from strong stimulation to strong inhibition.(46) The presence of 5-methylcytosine bases could induce differential conformational alterations of 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin-DNA cross-links and affect repair protein recognition and NER.

DNA methylation may also modulate repair at the nucleosome level in vivo, since it affects the affinity and positioning of histone octamer.(12,47,48) Addition of a 5-methyl group on cytosine changes the rotational positioning compared with normal nucleosomes. Moreover, DNA methylation and histone post-translational modification are closely related. In vivo, DNA methylation is always accompanied with genetically inactive heterochromatin, either by recruiting HDAC or by interacting with MBD family proteins.(49,50) DNA methylation often leads to histone deacetylation, whereas histone acetylation prevents DNA methylation.(4,51) These processes potentially would alter the
accessibility of a cisplatin-DNA lesion on the surface of histone octamer.(48) Previous studies revealed that NER of cisplatin-DNA adducts is modulated by the nucleosome core particle and by post-translational modifications of the histones.(13) DNA environmental changes caused by DNA methylation would add another layer of control on DNA repair from nucleosomes. Therefore, it would be interesting to investigate how DNA methylation would affect repair of cisplatin-damaged DNA at the nucleosomal level.

**Implications in Cancer and Processing of Cisplatin.** The present results provide insight into the possible activity of cisplatin in targeting certain malignant cells. The presence of 5-methylcytosine residues inhibits the formation of platinum-DNA adducts. Therefore, a DNA region that is deficient in 5-methylcytosine residues is more susceptible to platination. In fact, most cancer cells exhibit global hypomethylation of DNA and hypermethylation in the promoter regions of certain genes compared to normal cells.(11,52) Such global hypomethylation might be important for the preferential killing of cancer cells by cisplatin. Hypomethylation may lead to an increase in the platinum-DNA adducts level over that in normal methylated DNA, which may contribute to the activity of this drug against certain cancers.

In addition, the presence of 5-methylcytosines modulates HMGB1 binding affinity to DNA having the platinum lesion. Increased HMGB1 levels increase cellular sensitivity to cisplatin.(53) Widespread hypomethylation would provide
another possible way to increase interaction of HMGB1 with cisplatin-modified DNA.

On the other hand, hypermethylation in promoter regions silences the gene expression. The promoters of several repair genes are hypermethylated in certain cancers, affecting repair activity. (11,54) Taken together, the combined effects of increased platinum binding, increased binding of HMGB1 and subsequent suppression of repair at hypomethylated regions and the silencing of repair genes or other genes important for cell function by hypermethylation may cause more efficient cell death under these conditions compared to cells with normal methylation patterns. DNA methylation thus adds another level of complexity in understanding the regulation of cellular processing of cisplatin.

The combination of epigenetic drugs with cisplatin could provide promising effects for clinical use. The DNA methylation inhibitors 2'-deoxy-5-azacytosine and 5-azacytosine exhibit synergistic anticancer activity with cisplatin. (55-57) In addition, co-treatment cisplatin with HDAC inhibitor increased the cisplatin-DNA adduct formation and cellular sensitivity to the drug. (58,59) DNA methylation and histone deacetylation are closely related. (60-62) A combination of the HDAC inhibitor, trichostatin A (TSA), with 2'-deoxy-5-azacytosine caused a synergistic reactivation of expression of silenced genes in cancer cells. (63,64) Further exploration of “cocktail” combination of DNA methylation inhibitors, HDAC inhibitors, and cisplatin would be worthwhile for more efficient anticancer therapy.
Acknowledgment

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*EMBO J.* **21**, 6865-6873


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Table 6.1. The Ion Exchange HPLC Gradient for Isolation of Platinated 16mer Products

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Buffer A</th>
<th>% Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>35</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

Buffer A consisted of 0.025 M ammonium acetate in 10% aqueous acetonitrile (pH = 6.0). Buffer B contained 0.025 M ammonium acetate and 1 M sodium chloride in 10% aqueous acetonitrile (pH = 6.0).

Table 6.2. Buffer Conditions for the Time Course of Platination of Single-Stranded 16mer Substrates by Ion Exchange HPLC.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Buffer A</th>
<th>% Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td>12.5</td>
<td>45</td>
<td>65</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>18</td>
<td>72</td>
<td>28</td>
</tr>
</tbody>
</table>

Buffer A consisted of 0.025 M ammonium acetate in 10% aqueous acetonitrile (pH=6.0). Buffer B contained 0.025 M ammonium acetate and 1 M sodium chloride in 10% aqueous acetonitrile (pH=6.0).
Table 6.3. Buffer Conditions for the Time Course of Platination of Double-
Stranded 14mer Substrates by Reverse Phase Ion Exchange HPLC.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Buffer A</th>
<th>% Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
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<td>0</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Buffer A consisted of 1 M ammonium acetate in 7.5% aqueous acetonitrile (pH = 6.0). Buffer B was 50% aqueous acetonitrile (pH = 6.0).

Table 6.4. Electrospray Mass Spectrometry Results for 16mer Substrates.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Mass (Calculated) (g/mol)</th>
<th>Mass (Experimental) (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16GTG</td>
<td>4720.1</td>
<td>4720.4 ± 0.5</td>
</tr>
<tr>
<td>16 GTG Pt</td>
<td>4949.1</td>
<td>4947.7 ± 1.0</td>
</tr>
<tr>
<td>16GTGMe</td>
<td>4748.1</td>
<td>4748.4 ± 0.6</td>
</tr>
<tr>
<td>16GTGMe-Pt</td>
<td>4977.1</td>
<td>4975.2 ± 0.9</td>
</tr>
<tr>
<td>16GG</td>
<td>4705.1</td>
<td>4706.0 ± 1.0</td>
</tr>
<tr>
<td>16GG-Pt</td>
<td>4934.1</td>
<td>4931.4 ± 0.5</td>
</tr>
<tr>
<td>16GGMe</td>
<td>4733.1</td>
<td>4733.5 ± 0.6</td>
</tr>
<tr>
<td>16GGMe-Pt</td>
<td>4962.1</td>
<td>4960.7 ± 0.6</td>
</tr>
</tbody>
</table>

Table 6.5. UV-vis and AA Spectroscopy Results for 16mer Substrates.

<table>
<thead>
<tr>
<th>DNA</th>
<th>[Pt]/[DNA] Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 GTG Pt</td>
<td>0.97</td>
</tr>
<tr>
<td>16GTGMe-Pt</td>
<td>1.00</td>
</tr>
<tr>
<td>16GG-Pt</td>
<td>1.00</td>
</tr>
<tr>
<td>16GGMe-Pt</td>
<td>1.09</td>
</tr>
</tbody>
</table>
Table 6.6. Extent of Platination for Single-Stranded 16mer Substrates.*

<table>
<thead>
<tr>
<th>Time</th>
<th>16 GG</th>
<th>16 GG Me</th>
<th>16 GTG</th>
<th>16 GTG Me</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>24.08±0.54%</td>
<td>17.57±1.60%</td>
<td>21.32±0.72%</td>
<td>18.96±0.10%</td>
</tr>
<tr>
<td>1 h</td>
<td>27.80±0.09%</td>
<td>21.36±1.49%</td>
<td>25.30±1.07%</td>
<td>22.10±0.04%</td>
</tr>
<tr>
<td>2 h</td>
<td>36.16±0.92%</td>
<td>26.84±2.24%</td>
<td>32.57±0.20%</td>
<td>27.05±0.22%</td>
</tr>
<tr>
<td>4 h</td>
<td>39.70±1.20%</td>
<td>31.31±2.78%</td>
<td>39.14±0.01%</td>
<td>33.33±0.48%</td>
</tr>
<tr>
<td>6 h</td>
<td>41.77±1.24%</td>
<td>33.64±1.90%</td>
<td>42.31±0.37%</td>
<td>35.97±0.78%</td>
</tr>
<tr>
<td>8 h</td>
<td>42.80±1.24%</td>
<td>34.81±2.06%</td>
<td>44.11±0.45%</td>
<td>37.83±0.77%</td>
</tr>
<tr>
<td>24 h</td>
<td>44.06±1.20%</td>
<td>35.75±3.00%</td>
<td>46.65±0.05%</td>
<td>40.31±0.87%</td>
</tr>
</tbody>
</table>

*Average of two experiments.

Table 6.7. Extent of Platination for Double-Stranded 14mer Substrates.*

<table>
<thead>
<tr>
<th>Time</th>
<th>14 CC</th>
<th>14 MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>13.16±0.02%</td>
<td>6.00±0.31%</td>
</tr>
<tr>
<td>1 h</td>
<td>19.77±1.29%</td>
<td>8.13±0.18%</td>
</tr>
<tr>
<td>2 h</td>
<td>28.08±1.61%</td>
<td>12.82±0.85%</td>
</tr>
<tr>
<td>4 h</td>
<td>30.43±1.08%</td>
<td>18.13±0.63%</td>
</tr>
<tr>
<td>6 h</td>
<td>30.95±1.14%</td>
<td>19.28±1.78%</td>
</tr>
<tr>
<td>8 h</td>
<td>30.30±1.55%</td>
<td>19.95±0.78%</td>
</tr>
<tr>
<td>24 h</td>
<td>30.80±1.51%</td>
<td>22.46±1.77%</td>
</tr>
</tbody>
</table>

*Average of two experiments.
**Figure 6.1.** Strategy for synthesizing site-specifically platinated 195DNA probes.

Five synthetic oligonucleotides and one bearing a 1,2-d(GpG) or 1,3-d(GpTpG) intrastrand platinum-DNA cross-link were annealed and ligated as shown.
Figure 6.2. HPLC trace of platination products of 16mer DNAs after 18 h incubation. Peaks corresponding to the desired platinated DNA are identified.
Figure 6.3. Denaturing PAGE analysis of Maxam-Gilbert sequencing of 16GG and 16GGMe substrates. The 16GG and 16GGMe probes were sequenced by chemical degradation. Each lane is labeled according to its contents. The C+T lane and C lane for the 16GG show two extra bands (arrows) that do not appear in the corresponding positions in the 16GGMe lanes. These differences indicate the presence of 5-methylcytosine residues in the 16GGMe sample at these locations.
Figure 6.4. Maxam-Gilbert sequencing results for the 16GG and 16GGMe. The intensities of bands in the gel were plotted as a function of distance to generate the plots. The 16GGMe has the same sequence as the 16merGG with the exception of the 5-methylcytosine residues. The 5-methylcytosine residues are not cleaved, and therefore do not produce bands in the gel (arrows).
Figure 6.5. Maxam-Gilbert sequencing of 16GTG and 16GTGMe substrates. The 16GTG and 16GTGMe were sequenced by chemical degradation. Each lane is labeled according to its contents. The C+T lane and C lane for the 16GTG show two extra bands (arrows) that do not appear in the corresponding positions in the 16GTGMe lanes. These differences are indicative of the presence of 5-methylcytosine residues in the 16GTGMe sample at these locations.
Figure 6.6. Maxam-Gilbert sequencing results for the 16GTG and 16GTGMe. The intensities of bands in the gel were plotted as a function of distance to generate the plots. The 16GTGMe has the same sequence as the 16GTG with the exception of the 5-methylcytosine residues that replace the cytosine residues adjacent to the platinum binding site. The 5-methylcytosine residues are not cleaved, and therefore do not show bands in the gel (arrows).
Figure 6.7. PAGE analysis of Maxam-Gilbert sequencing of 36mer substrates. The 36mers were sequenced by chemical degradation. Each lane is labeled according to its contents. The C+T lane and C lane for the 36CC show two extra bands that do not appear in the corresponding positions in the 36MM lanes. Similar differences can be seen between the 36CAC and 36MAM lanes. These differences are indicative of the presence of 5-methylcytosine residues in the 36MM and 36MAM sample at these locations.
Figure 6.8. Maxam-Gilbert sequencing results for the 36CC and 36MM probes. The intensities of bands in the gel were plotted as a function of distance to generate the plots. The 36MM has the same sequence as the 36CC with the exception of the 5-methylcytosine residues that replace the cytosine residues located 18 and 19 nucleotides from the 5' end. The 5-methylcytosine residues are not cleaved, and therefore do not show bands in the gel (arrows).
Figure 6.9. The Maxam-Gilbert sequencing results for the 36CAC and 36MAM. The intensities of bands in the gel were plotted as a function of distance to generate the following plots. The 36MAM has the same sequence as the 36CAC with the exception of the 5-methylcytosine residues that replace the cytosine residues located 18 and 20 nucleotides from the 5' end. The 5-methylcytosine residues are not cleaved, and therefore do not show a band in the gel.
Figure 6.10. Comparison between platinated and non-platinated substrate of Maxam-Gilbert sequencing. In order to verify the presence of platinum in the 16GG Pt substrate, the products from the G reaction were compared from this substrate with the products from the G reaction from the 16GG non-platinated probe. Although the background noise in these two samples is the same, the intensity of the bands for the 16GG are nearly two-fold more intense than the corresponding bands for the 16GG-Pt sample. This suppression of signal can be attributed to the presence of a platinum adduct in the 16GG-Pt samples. The dotted line above is the data from the 16GG and the solid line represents the data from the 16GG-Pt sample.
Figure 6.11. Time course of platination of 16GG and 16GGMe. The extent of platination was evaluated at regular intervals. The 16GG (circles) is more rapidly platinated than its methylated analog 16GGMe (squares).
Figure 6.12. Time course of platination kinetics of 16GTG and 16GTGMe. The extent of platination was evaluated at regular intervals. The 16GTG (circles) is more rapidly platinated than its methylated analog 16GTGMe (squares).
Figure 6.13. Time course of platination kinetics of ds14CC and ds14MM. The extent of platination was evaluated at regular intervals. The ds14CC (circles) is more rapidly platinated than its methylated analog ds14MM (diamonds).
Figure 6.14. EMSA of ds16mers with HMGB1-dom A. Methylation at cytosines flanking the GG-Pt adduct slightly reduced the binding of HMGB1 dom A, whereas methylation at cytosines on the strand opposite GG-Pt lesion significantly reduced protein binding. B. Quantitative EMSA results of HMGB1-dom A with ds16GG (red), ds16GGMe (blue), and ds16GG//Me (green).
Figure 6.15. A. Non-denaturing PAGE gel of ds195mers. B. Excision assay with methylated and non-methylated DNA probes. Lane 1, 195GTG-Pt Me; lane 2, 195GTG-Pt//MAM; lane 3, 195GTG-Pt//CAM; lane 4, 195GTG-Pt//MAC; lane 5, 195GTG-Pt; lane 6, 195GG-Pt Me; lane 7, 195GG-Pt//MM; lane 8, 195GG-Pt//CM; lane 9, 195GG-Pt//MC; lane 10, 195GG-Pt. C. The relative excision efficiencies of ds 195mers. Data in columns 1-5 are normalized by the excision efficiency of column 5, data in columns 6-10 are normalized by the excision efficiency of column 10.
Appendix 6.1

The following twelve oligonucleotides were synthesized for use as the 195mer repair substrates. 5-Methylcytosine residues are represented with the letter M.

83mer:
GCTGACAACAAAGATTGTCTTTTCTGACCAGATGGACGCGGCCACCC TCAAAGGCATCACCGCGGGCCAGGTGAATATCA-3'

16mer GG: 5'-CCTCTCCGGCCTCTCC-3'
16mer GTG: 5'-CCTCTCCGTGCTCTCC-3'
16mer GG Me: 5'-CCTCTTCMGGMCTCTCC-3'
16mer GTG Me: 5'-CCTCTTCMGTGMTCTCC-3'

96mer:
5'-GTATTATGAATTCCAGCTGCTCGAGCTCAATTAGTCAGACCCCA CAGCTGGGAGAGAACAGCGACCCCGCGGGCCCCGGCAGGCGGCTCAAGCA GGAG-3'

87mer: GCTCCTGCTTGAGCCGCCTGCCGGGGCCCGCGGGTCGCTGTTCTCT CCAGCTGTGTTGGGTCTGACTAATTGAGCTCGAGCAGCTGAA-3'

36mer CC: 5'-TTCATAATACGGAGAGGCCGGAGAGGTGATATTCAC-3'
36mer MM: 5'-TTCATAATACGGAGAGMAMGAGAGGTGATTATTCAC-3'
36mer CAC: 5'-TTCATAATACGGGAGAGCACGGAGAGGTGATTATTCAC-3'
36mer MAM: 5'-TTCATAATACGGAGAGMAMGAGAGGTGATTATTCAC-3'

72mer: CTGGCCCGCGGTGATGCCTTGAGGGTGGCCGCGTCCATCTGGTC AGAAAAGACAAATCTTTTTGGTCAAG-3'

The following two self-complementary sequences were synthesized for the kinetic study of platinating a double-stranded DNA:

14mer CC: 5'-TATAGGTACCTATA-3'
14mer MM: 5'-TATAGGTAMMTATA-3'
Chapter 7 Transcription-Coupled and DNA Damage-Dependent Ubiquitination of RNA Polymerase II in Vitro*

* The work in this chapter was published in PNAS 99, 4239-4244 (2002).
Introduction

Transcription-coupled repair (TCR) is a cellular pathway for the removal of the many lesions that block and arrest transcription. TCR is responsible for the rapid and preferential repair of damage in the transcribed strand of active genes (1-3). Specific mutations in genes are known to cause defects in TCR, including Cockayne syndrome (CS) complementation groups A and B which debilitate TCR of UV- and oxidative-damage induced lesions (4, 5), and the breast cancer susceptibility gene BRCA1, which is defective in TCR of oxidative DNA damage (6, 7). Lesions induced by chemotherapeutic agents such as cisplatin are also removed by TCR (8, 9), implicating TCR in the differential response of cells to cancer treatment.

The recent discovery that UV irradiation or cisplatin treatment of cells induces ubiquitination of the large subunit of RNA Pol II (Pol II LS) and its degradation (10, 11), suggested a biochemical link between ubiquitination and DNA repair. The ubiquitinated polymerase is hyperphosphorylated on the C-terminal domain (CTD) of Pol II LS (10). Ubiquitination of Pol II was not observed in TCR-deficient CSA and CSB cells but could be restored upon transfection of their respective cDNAs (10). These results further indicate a relevance of Pol II ubiquitination to the mechanism of action of cisplatin. This chemotherapeutic agent is routinely used in cancer treatment against otherwise resistant solid tumors, such as encountered in testicular, ovarian and breast cancer (12).

Interestingly, Pol II undergoes degradation in cells treated with alpha-amanitin, an inhibitor of its transcription (13). This amatoxin binds specifically to RNA Pol II (14,
15), and arrests the elongating polymerase in a conformation that inhibits incorporation of a subsequent nucleotide triphosphate to the nascent RNA transcript (16, 17). Pol II transcription inhibition by alpha-amanitin may resemble its transcription arrest at a DNA lesion. It is not known whether alpha-amanitin dependent degradation of Pol II is mediated by the ubiquitin pathway.

In order to understand the cellular response to DNA damage during transcription, we have investigated transcription- and DNA damage-dependent ubiquitination of RNA Pol II in vitro. We significantly modified the original in vitro assay for ubiquitination of Pol II (18) to increase its sensitivity. In addition, we compared nuclear extracts from cells at different cell cycle stages in order to optimize ubiquitination activity.

Materials and Methods

Chemical Reagents
Alpha-amanitin and His-tagged ubiquitin (His-Ub) were purchased from Calbiochem. Aphidicolin, hydroxyurea and nocodazole (methyl-5-[2-thienyl-carbonyl]-1H-benzimidazol-2-yl) carbamate) were purchased from Sigma-Aldrich. PALA (N-phospho-N-acetyl-L-aspartate) was obtained from the Drug Biosynthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Cisplatin was provided by Johnson Matthey.
Preparation of Nuclear Extracts

Nuclear extracts were prepared from HeLa cells as described (19). Cells were maintained in MEM with 10% horse serum (Gibco BRL) at 37°C at a cell density of 0.5 - 1.0 x 10⁶ cells/mL. To arrest HeLa cells at different stages, cells were either grown in spinner flasks to 0.5 x 10⁶ cells/mL as above, or on plates in DME containing 10% heat-inactivated fetal bovine serum (Gibco BRL) to 30-50% confluency. The cells were then serum-starved for 16 h by replacing the serum-containing media with serum-free media. Serum was added back to the media to a final concentration of 10% on the following day, and cells were incubated for a further 8 h prior to the addition of various drugs to a final concentration of 1 mg/mL aphidicolin, 750 μM hydroxyurea, 2 μg/mL nocodazole and 500 μM PALA, respectively. Cells were incubated with the drugs for 16 h before harvesting.

For analysis of drug-treated cells, 1 x 10⁶ cells in PBS were permeabilized with 95% ethanol and stained by incubating with RNase I (200 μg/mL, Calbiochem) and propidium iodide (25 μg/mL, Sigma-Aldrich). The stained cells were analyzed in a fluorescence-activated cell sorter (FACS Calibur, Becton Dickinson) and cell cycle stages determined with the MODFIT LT program.

Platination of Plasmid DNA

Plasmids pUC19 (New England Biolabs), pG5MLP-G380 (obtained from D. Tantin), pUC118-296 (obtained from C. Kneip), pHIV+TAR-G400 and pHIVΔTAR-G100 (20) were prepared from overnight cultures of plasmid-harboring JM109 (Promega) in
LB media using a Promega Wizard Midi-prep kit. pG5MLP-G380 contained the major late promoter (MLP) with a 380 bp G-less cassette inserted 10 bp downstream of the initiation site. pUC118-296 contained the MLP and 5S rDNA nucleosome positioning sequences (21).

The cis-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ cation (activated form of cisplatin) was prepared by stirring a solution of cis-DDP (6.4 mg, 0.021 mmol) with 1.98 molar equivalent AgNO$_3$ overnight in the dark. Any precipitated AgCl was removed by centrifugation, and the supernatant was diluted to a final concentration of 1 mM cis-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ in water. Platination of plasmids was achieved by incubating 0.043 nmol DNA with activated cis-DDP at $r_t = 0.1$ (where $r_t$ equals the number of moles of drug added per mole of DNA nucleotide) in 100 μL 10 mM sodium phosphate (pH 6.8) for 16 h at 37 °C. The unbound platinum salt was removed by dialysis. The ratio of platinum bound per nucleotide, i.e. the $r_b$ value, was determined by flameless atomic absorption spectroscopy (Perkin-Elmer HGA-800 Analyst 300) and UV absorption spectroscopy (Hewlett Packard 8453).

In Vitro Transcription Assay

Transcription reactions (25 μL) were performed essentially as described (20). Nuclear extracts (30 μg) were incubated at 30°C for 30 min in buffer 1 [10 mM HEPES-KOH (pH = 7.9), 10% glycerol (v/v), 60 mM KCl, 7 mM MgCl$_2$, 0.1 mM EDTA, 1 mM ATP, 10 mM creatine phosphate, 12 μg/mL poly I-C, 2 μg/mL poly dG-dC] containing 7 mM DTT, C/G/UTP mix (25:200:200 μM), 10 μCi [α-$^{32}$P]CTP [800 Ci (29,600
GBq)/mmol, and 200 ng DNA template. To study the effect of alpha-amanitin, the reactions were pre-incubated at 30°C for 15 min in the absence and presence of 5 μM alpha-amanitin under the above conditions, but with the NTPs and creatine phosphate added subsequently. The reactions were then incubated for a further 30 min. [In order to observe a complete inhibition of transcription by alpha-amanitin, the pre-incubation step was included to allow for sufficient drug binding to Pol II. For the in vitro ubiquitination assay (see below), the results obtained with or without pre-incubation with alpha-amanitin were similar.] All reactions were subjected to RNase T1 digestion at 37°C for 5 min and G-less RNA transcripts were isolated and analyzed by urea-PAGE as described (22). The gels were vacuum-dried and autoradiographed with KODAK X-O{MAT film at -80°C for 24-40 h.

In Vitro Ubiquitination Assay

Reactions were set up as in in vitro transcription assays with 60-70 μg nuclear extract in buffer 1 (which contains 1 mM ATP) with 1 μg DNA template, 200 μM CTP, UTP and GTP and 1.25 μg His-tagged ubiquitin (His-Ub). Saturating levels of alpha-amanitin (5 μM) were added to the mix where indicated. Reactions were incubated at 30°C for 45 min, and His-ubiquitinated proteins were isolated by incubating at 4°C for 1 h with 20 μl Ni-NTA agarose (Qiagen) in a final volume of 200 μL in buffer 2 [50 mM sodium phosphate (pH 7.9), 0.3 M NaCl, 0.05% Tween 20 (v/v)] containing 10 mM imidazole. The Ni-NTA agarose was pre-blocked with 1 mg/mL BSA prior to use.
After low-speed centrifugation (735 g), the Ni-agarose beads containing His-ubiquitinated proteins were washed twice with 1 mL buffer 2 containing 50 mM imidazole. The Ni-bound proteins were eluted with SDS-loading buffer [20 mM Tris-HCl (pH 6.8), 10% glycerol (v/v), 100 mM 2-mercaptoethanol, 1% SDS (w/v), 0.02% bromophenol blue (w/v)] containing 0.1 M EDTA (pH 7.0). To analyze the protein content, samples were boiled in SDS-loading buffer and electrophoresed in SDS-7.5% polyacrylamide gels, followed by electro-transfer to Immobilon-P membranes (Millipore).

Pol II was detected with N20 (Santa Cruz), a non-phospho-specific rabbit polyclonal IgG. The phosphorylated form of Pol II was detected with H5 or H14 (Covance), a mouse monoclonal IgM which recognizes phospho-serine 2 or 5 of the CTD heptapeptide repeat.

Results

Alpha-amanitin Stimulates Ubiquitination of RNA Pol II

To investigate a possible correlation between ubiquitination of RNA Pol II and the arrest of transcription, we examined the effect of alpha-amanitin, a Pol II inhibitor, on polymerase ubiquitination in vitro. Previous results suggested little or no effect of alpha-amanitin on in vitro ubiquitination of polymerase in nuclear extracts from unsynchronized cells (18). We prepared nuclear extracts from cells arrested in G1/S phase by aphidicolin, a DNA α-Pol inhibitor (23), and tested for alpha-amanitin dependent ubiquitination of Pol II LS (see below for drug and cell cycle dependence).
Cell cycle stages were verified by FACS analysis (not shown). To increase the sensitivity of the assay, His-Ub was added to the reaction, and ubiquitinated proteins were selected on Ni-NTA agarose (Qiagen) and analyzed by Western blotting. The reaction conditions were those used for transcription (see Material and Methods).

SDS-PAGE of total unselected extracts and Western blot analysis with a non-phospho-specific antibody to the N-terminus of Pol II LS (N20) revealed that hypophosphorylated Pol II_A (Figure 7.1a, lane 1) was converted to hyperphosphorylated Pol II_0 during the reaction (Figure 7.1a, lanes 2 and 4). Upon addition of alpha-amanitin, a new slow migrating band appeared (marked with an asterisk; Figure 7.1a, lanes 3 and 5). This slow migrating band was observed in the Ni-bound fraction only when His-Ub was present in the reaction (Figure 7.1a, lane 10), indicative of ubiquitination of RNA Pol II. The Pol II_A and II_0 bands recovered in the Ni-NTA fractions in the absence of His-Ub are due to non-specific binding to Ni agarose (Figure 7.1a, lanes 7-8). When the Western blot was stripped and re-probed with an antibody to the phosphorylated CTD (H14), ubiquitinated Pol II was also detected (marked with an asterisk; Figure 7.1b, lanes 3, 5 and 10). H14 recognizes phospho-serine 5 of the CTD heptapeptide repeat. Similar results were obtained using H5, another antibody which specifically recognizes phospho-serine 2 of the CTD repeat (not shown). Thus alpha-amanitin stimulates ubiquitination of Pol II LS, and hyperphosphorylated II_0 comprise the ubiquitinated species, consistent with previous results (10). Only a small percentage of Pol II (~5%) is ubiquitinated since, relative to
the input material (Figure 7.1a and b, lanes 1-5), most of the polymerase was recovered in the fraction not bound to the column (flow-through) (Figure 7.1a and b, lanes 11-15).

To determine whether ubiquitination activity varied at different stages of the cell cycle when arrested by drug treatment, we compared the activities of extracts from cells treated with different cell-cycle inhibitors. The fraction of cells at each stage was determined by FACS analysis (not shown). Extracts from cells treated respectively with aphidicolin, hydroxyurea (a ribonucleotide reductase inhibitor) (24) and PALA (an inhibitor of de novo pyrimidine biosynthesis) (25), all showed similar levels of alpha-amanitin induced ubiquitination (Figure 7.2a, lane 5-6, 8-9, and 11-12, respectively). Consistent with previous findings (18), extracts from unsynchronized cells did not show an appreciable alpha-amanitin induction of ubiquitination (Figure 7.2a, lanes 2-3). G2/M extracts from cells treated with the microtubule inhibitor, nocodazole (26), gave a higher proportion of Pol IIo to Pol II A under the reaction conditions (Figure 7.2a, total, lanes 13-15), but showed little or no ubiquitination (Figure 7.2a, lanes 14-15). In parallel work, we compared in vitro transcription activities of the different extracts using pG5MLP-G380, which contains a major late promoter (MLP) and a 380 bp G-less cassette (G380). The amount of G380 transcript produced by G1/S and S extracts after RNase T1 digestion was approximately two-fold higher (Figure 7.2b, lanes 3 and 5) than extracts from unsynchronized cells (Figure 7.2b, lane 1), whereas that of G2/M extracts was considerably lower (Figure 7.2b, lane 7). In all cases, alpha-amanitin inhibited transcription as judged by disappearance of the transcript (Figure 7.2b, lanes 2, 4, 6, and 8).
Dependence of Ubiquitination on DNA

Since alpha-amanitin binds to Pol II itself (15, 27), we studied whether ubiquitination of polymerase due to alpha-amanitin depends on the presence of DNA. We titrated the ubiquitination reaction against increasing concentrations of template DNA (Figure 7.3). In the absence of DNA, only low-level or background ubiquitination of polymerase was observed (Figure 7.3, lanes 3 and 4). When a promoter-containing DNA (pUC118-296) was present, the extent of ubiquitination with alpha-amanitin increased with increasing amounts of DNA until 1.2 μg template was added to the reaction (Figure 7.3, lanes 5-10). The level of ubiquitination, however, declined upon further addition of DNA (Figure 7.3, lane 12). Therefore, alpha-amanitin induces ubiquitination dependent upon the presence of DNA, probably by interacting with polymerase-DNA complexes during transcription. This stimulation could not be recapitulated by nucleotide depletion or termination of transcription by addition of 3'-O-methyl-GTP (not shown). At high DNA concentrations in vitro, total transcription by Pol II is typically not promoter-dependent and occurs in the absence of a promoter (not shown). Consistent with this behavior, duplex plasmid DNA stimulated alpha-amanitin dependent ubiquitination independent of the promoter sequence (not shown). Similarly, both supercoiled and linearized plasmid were as effective in stimulating ubiquitination (not shown).
**Ubiquitination is Induced by Arrest of Transcription on Cisplatin-Damaged DNA**

The above data strongly suggest that polymerase-DNA complexes stalled during transcription are targeted for ubiquitination. To investigate a role for DNA damage in stimulating Pol II ubiquitination, DNA templates containing lesions that arrest transcription by Pol II, were added to the reaction. Plasmid DNAs modified by cisplatin having an $r_b$ value (bound platinum to DNA nucleotide ratio) of 0.055, or an average of one platinum adduct per 18 DNA bases, were tested for induction of Pol II ubiquitination. Reactions containing alpha-amanitin were also performed in parallel for comparison of activities.

As observed earlier, ubiquitination of Pol II LS was negligible in a reaction lacking DNA or containing unmodified DNA (Figure 7.4a, lanes 2-4). In contrast, ubiquitination was induced significantly by each cisplatin-modified template (Figure 7.4a, lanes 6, 9 and 12), to similar levels as that induced by alpha-amanitin with unmodified DNA (Figure 7.4a, lanes 5, 8 and 11). This result indicates that both cisplatin and alpha-amanitin induce ubiquitination in a similar fashion, probably by inhibiting elongation of Pol II transcription on DNA. At this level of cisplatin modification, transcription from cisplatinated DNA containing a 380 bp G-less cassette (G380) was abolished, whereas transcription from an unmodified DNA template with a 100 bp G-less cassette (G100) was unaffected in the same reaction (Figure 7.4b, lanes 1-2). Thus, the absence of transcript from the G380 cassette is specific to cisplatin modification of the DNA, suggesting that ubiquitination is induced by arrest of transcription due to the lesions in the DNA. As with alpha-amanitin, ubiquitination
due to cisplatin-modified DNA is independent of the presence of a specific promoter on the template DNA. Ubiquitination was observed with cisplatinated pUC19 DNA, albeit at a lower level (Figure 7.4a, lanes 11-12).

**Dependence of Ubiquitination on Transcription**

The transcription dependence of the ubiquitination reactions was examined further by using antibodies to a general transcription factor involved in pre-initiation complex assembly, viz. TFIIB (28). Addition of an antibody to TFIIB inhibited transcription (Figure 7.5c, lane 3), as indicated by disappearance of the transcript present in a normal *in vitro* transcription reaction (Figure 7.5c, lane 1). As expected, addition of an antibody to c-myc had no effect on transcription (Figure 7.5c, lane 2). When the ubiquitination reaction was tested in the presence of an anti-TFIIB antibody, the ability of either alpha-amanitin or cisplatin-modified DNA to stimulate ubiquitination was severely reduced (Figure 7.5a and b, bound fractions, lanes 6-7) relative to a control without antibody (Figure 7.5a and b, bound fractions, lanes 2-3). Ubiquitination overall was unaffected by an antibody to c-myc (Figure 7.5a and b, bound fractions, lanes 4-5). Analysis of the total extracts revealed that phosphorylation of CTD was largely inhibited by addition of antibody to TFIIB (Figure 7.5a and b, total, lanes 6-7). This behavior is probably a consequence of inhibition of transcription initiation, which is accompanied by Pol II phosphorylation.
Discussion

We report the first evidence for transcription-coupled and DNA damage-dependent ubiquitination of RNA Pol II in vitro. Addition of alpha-amanitin, an inhibitor of Pol II elongation, stimulates ubiquitination of Pol II LS. Hyperphosphorylated Pol II is ubiquitinated, consistent with an engagement in elongation. This stimulation requires the addition of template DNA, also indicating a dependence upon polymerase being engaged in transcription. Moreover, addition of DNA templates containing cisplatin adducts, which arrest transcription, stimulates ubiquitination of Pol II LS as compared with reactions containing unmodified templates. The transcription dependence of both alpha-amanitin and cisplatin-induced ubiquitination was further demonstrated by the abrogation of ubiquitination following addition of antisera to a component of the transcription pre-initiation complex, TFIIB.

Implications for Transcription-Coupled DNA Repair

Previous in vivo experiments pointed to a possible role for ubiquitination of Pol II in transcription-dependent DNA repair. Bregman et al. (10) observed that the polymerase was ubiquitinated upon DNA damage from UV irradiation or cisplatin treatment of repair-competent cells and that this ubiquitination was absent in TCR-deficient Cockayne syndrome cells. Our finding that ubiquitination is triggered by alpha-amanitin as well as cisplatin-DNA adducts in vitro, both of which arrest transcription, is consistent with a role for ubiquitination in the cellular response to DNA damage. The transcription dependence of ubiquitination in vitro suggests that
components of the reaction specifically recognize a form of the polymerase generated by its arrest at lesions or by alpha-amanitin. This same arrested complex is probably a key structure signalling TCR.

Only a small proportion of polymerase is ubiquitinated (~5%) in a reaction containing saturating levels of alpha-amanitin. Strikingly, the levels of ubiquitination induced by alpha-amanitin and by DNA with an average of one platinum adduct per 18 DNA bases (approximately 1 platinum adduct per helical turn) are qualitatively similar. Alpha-amanitin binds with high affinity ($10^{-10} - 10^{-8}$ M) (15) to a conserved region of Pol II LS (27). It is a very specific and potent inhibitor of the elongating polymerase (14, 29). Biochemical studies reveal that alpha-amanitin neither dissociates the polymerase-DNA-RNA complex nor competes with nucleotide incorporation (14, 15). Rather, under conditions of elongation, it blocks Pol II after formation of a phosphodiester bond, and probably inhibits a transition in the conformation of Pol II required for its movement along the DNA template (16, 17). Thus the binding of alpha-amanitin may arrest the elongating polymerase in a transitional conformation that is recognized by components in the reaction specifying ubiquitination. Based on the similarities in ubiquitination of Pol II due to alpha-amanitin and cisplatin-DNA damage, the platinum lesion most likely also blocks translocation of Pol II along the DNA, stabilizing a similar transitional conformation. The major cisplatin-DNA adduct is the intrastrand cross-link between adjacent purine residues, with the 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links being the predominant lesions (30). Both the 1,2-d(GpG) and 1,3-d(GpTpG) adducts are efficient blocks to transcript elongation in vitro (31, 32) and in vivo (33).
Evidence suggests that the inability to remove an RNA polymerase stalled at a DNA lesion prevents access to repair enzymes, thus enhancing the mutation rate. The same polymerase would also block elongation by other polymerases and hence interfere with gene expression. An RNA Pol II stalled at a UV-induced cyclobutane pyrimidine dimer prevented access of a small bacterial repair protein \textit{in vitro} (34). Human mutant cells impaired in TCR of oxidative lesions, \textit{viz.} CSA and CSB, as well as subgroups of xeroderma pigmentosum XPB, XPD and XPG that show Cockayne syndrome, exhibit a high mutation frequency of 30-40% at an 8-oxoguanine lesion compared with a normal level of 1-4% (35). TCR of oxidative lesions, including thymine glycols, is also defective in cells derived from \textit{brca1-} mice (6) and the human breast carcinoma cell line HCC1937 (7, 36), which harbors a mutation of the BRCA1 gene inherent in many incidences of breast and ovarian cancer (37, 38). Transcription was not observed beyond the 8-oxoguanine lesion on plasmid DNA transfected into the XPG/CS and HCC1937 cells (35, 36). Further, TCR-deficient CSA and CSB cells are more susceptible to apoptosis upon UV irradiation, perhaps due to the inefficient removal of RNA Pol II at UV-induced lesions and subsequent blockage of transcription (39).

\textit{The Cellular Signals Triggering Ubiquitination}

Ubiquitination occurs by covalent attachment of multiple ubiquitin molecules to the protein substrate. This process is typically mediated by three enzymes, the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and the substrate-
specific ubiquitin ligase complex E3 (40, 41). Ubiquitination by E3 ligase is highly regulated and in many cases, substrate recognition requires covalent modification, e.g. in the phosphorylation of IκB (42) and β-catenin (43). Immunoblot analyses of whole cell extracts from UV-irradiated/cisplatin-treated cells (10) and of Ni-selected nuclear extracts from the in vitro ubiquitination assay (this work), demonstrate that ubiquitinated RNA Pol II is recognized by antibodies specific to phosphorylation of either serine 2 or serine 5 of the CTD. Previous results using nuclear extracts from growing cells indicated that a GST-CTD fusion protein was ubiquitinated in a kinase-dependent manner (18), suggesting some modification of this repetitive domain may be important for ubiquitination.

An interesting finding of the present work is that nuclear extracts from cells arrested with drugs which affect DNA synthesis in the G1/S phase, exhibit significantly enhanced ubiquitination activity relative to unsynchronized cells. The reason for this behavior is unclear since ubiquitination of Pol II following DNA damage is observed in vivo in unsynchronized cells (10), which are primarily in the G1 phase of the cell cycle. Of possible relevance is that all three drug inhibitors activate or induce proteins involved in repair of DNA damage. Included are the DNA damage cell cycle checkpoint protein p53 (44-47), and the breast cancer susceptibility protein BRCA1 (48), which in addition to transcription-coupled repair of oxidative damage (6, 7, 36), is involved in double-strand break DNA repair (49, 50). p53 interacts with proteins involved in TCR, viz. CSB, as well as the XPB and XPD subunits of TFIIH (51), with the latter interactions being important for induction of apoptosis (52). BRCA1, on the other
hand, undergoes phosphorylation and relocalized into replicating DNA structures upon treatment of S-phase cells with hydroxyurea or DNA damage agents (48). BRCA1 also interacts with RNA Pol II holoenzyme (53) and contains a ring finger domain that interacts with the ring finger domain of another protein BARD1 (54). Ring finger domains are generally involved in the ubiquitination pathway (55) and BRCA1 can facilitate E2-dependent ubiquitination \textit{in vitro} (56). BARD1, which co-localized with BRCA1 upon DNA damage (48), enhances ubiquitination activity; a breast cancer-derived Cys mutation in the ring finger of BRCA1, which is deficient in its interaction with BARD1, abolishes this activity (57). The same mutation is defective as a tumor suppressor. The substrate for ubiquitination by BRCA1 is currently not known but may be RNA polymerase II [also see (58)].

\textit{Implications for the Cisplatin Mechanism of Action}

Previous studies revealed that specific architectural changes induced upon formation of the major cisplatin 1,2-intrastrand DNA cross-links lead to binding of minor groove intercalating proteins including HMGB1 (59, 60) and TBP (61). Such processes inhibit nucleotide excision repair (NER) \textit{in vitro} (62) and delay NER \textit{in vivo} (63), leaving the adducts intact for subsequent recognition through arrest of Pol II elongation during TCR. The present results suggest that ubiquitination of RNA Pol II may be an important event in the repair of cisplatin damage. Because removal of the stalled polymerase is critical for repair, inhibition of ubiquitination of Pol II is likely to enhance cell killing by cisplatin.
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References


Figure 7.1. Ubiquitination of Pol II is induced by alpha-amanitin in an in vitro assay. Nuclear extracts from cells treated with aphidicolin were incubated in an in vitro ubiquitination reaction with pUC118-296 in the absence or presence of His-Ub and alpha-amanitin (α-am). His-ubiquitinated proteins were selected on Ni-NTA agarose. Total (lanes 1-5), Ni-bound (lanes 6-10), and flow-through fractions from the Ni-NTA agarose (lanes 11-15) were analyzed by SDS-7.5% PAGE and transferred to a PVDF membrane. The immunoblot was probed consecutively with antibodies to, (a) the N-terminus of RNA Pol II (N20), and (b) the phosphorylated CTD of Pol II (H14). Lanes 1, 6 and 11 show untreated nuclear extracts.
Figure 7.2. Comparison of ubiquitination and transcription activities of various drug-induced cell cycle stage nuclear extracts. (a) The in vitro ubiquitination assay was performed as described in Figure 7.1 with nuclear extracts from unsynchronized cells (lanes 1-3) as well as with cells treated respectively with aphidicolin (lanes 4-6), hydroxyurea (HU) (lanes 7-9), PALA (lanes 10-12), and nocodazole (lanes 13-15). Reactions (containing His-Ub) were analyzed by SDS-7.5% PAGE, and the immunoblots for the total (top) and Ni-bound proteins (bottom) are shown. The immunoblot of total extracts is shown at a lower exposure time than that for the bound proteins to allow better resolution of protein bands. Lanes 1, 4, 7, 10 and 13 show untreated nuclear extracts. (b) Nuclear extracts from unsynchronized cells (lanes 1 and 2), and cells arrested at the cell cycle stages of G1/S (lanes 3 and 4), S (lanes 5 and 6) and G2/M (lanes 7 and 8), were incubated in an in vitro transcription reaction with pG5MLP-G380 containing a 380 bp G-less cassette (G380), in the absence and presence of alpha-amanitin respectively. Reactions were subsequently treated with RNase T1, and G-less transcripts were isolated and analyzed by 8 M urea-6% PAGE. An autoradiograph of the gel is shown.
Figure 7.3. Alpha-amanitin induction of Pol II ubiquitination is DNA-dependent. Ubiquitination reactions with nuclear extracts from aphidicolin-treated cells and His-Ub were titrated against increasing amounts of pUC118-296 (0-2.4 µg, lanes 3-12), and the effect of alpha-amanitin (α-am) was assayed. Reactions were analyzed as described in Figure 7.1, and immunoblots of total (top) and Ni-NTA bound proteins (bottom) against N20 are shown. Lanes 1, untreated extracts. Lanes 2, ubiquitination reaction containing 1 µg DNA, incubated in the absence of both His-Ub and alpha-amanitin.
Figure 7.4. Ubiquitination of Pol II is induced to a similar extent by cisplatin-modified DNA and alpha-amanitin. (a) The effects of alpha-amanitin and various cisplatin-modified DNAs on Pol II ubiquitination were examined in the \textit{in vitro} ubiquitination assay. Immunoblots of total (top) and bound proteins (bottom) are shown. Lanes 1, untreated nuclear extracts. Lanes 2 and 3, ubiquitination reactions performed in the absence of DNA, with alpha-amanitin ($\alpha$-am) added where indicated. Lanes 4-5, 7-8 and 10-11, reactions containing unmodified DNA with the addition of alpha-amanitin where indicated. Lanes 6, 9 and 12, reactions containing cisplatinated DNA (cis). (b) \textit{In vitro} transcription reactions were performed simultaneously with pG5MLP-G380 containing a G380 cassette and pHIVATAR-G100 containing a G100 cassette. Lane 1, transcription with unmodified DNA. Lane 2, transcription with cisplatinated pG5MLP-G380 but unmodified pHIVATAR-G100. Autoradiograph of gel is shown.
Figure 7.5. Stimulation of polymerase ubiquitination by alpha-amanitin or cisplatin-modified DNA is suppressed by inhibiting transcription. (a) A typical ubiquitination reaction was carried out in the absence (lane 2) or presence of alpha-amanitin (α-am) (lane 3). Corresponding reactions were performed with the addition of antibodies to c-myc (lanes 4-5) or TFIIB (lanes 6-7). Figure shows immunoblots of total extracts (left) and Ni-bound fractions (right), which were developed with antibodies against Pol II LS (N20). The immunoblot of total extracts is shown at a lower exposure time than that for the bound proteins. Lanes 1 show untreated nuclear extracts. (b) Ubiquitination reactions were performed in the presence of unmodified DNA (lane 2) or cisplatin-modified DNA (cis-DDP) (lane 3). Corresponding reactions were conducted with the addition of the different antibodies, followed by immunoblotting as described in (a). Lanes 1 show untreated nuclear extracts. (c) In vitro transcription reactions using pG5MLP-G380 were performed in the absence (lane 1) and in the presence of an antibody to c-myc (lane 2) or TFIIB (lane 3). Reactions were analysed as in Figure 7.2b. Autoradiograph of gel is shown.
Part II. Synthesis of Site-Specifically Cisplatin-Modified DNA Templates and In Vitro Transcription Study
Introduction

Cisplatin, *cis*-diaminedichloroplatinum (II), is routinely used for treatment of testicular, ovarian, breast and several other types of cancers. DNA is widely accepted as the biologically relevant target of cisplatin. Cisplatin forms covalent, bifunctional DNA adducts, in which platinum binds to the N(7) positions of purine nucleotides, forming 1,2-d(GpG) and 1,2-d(ApG) intrastrand, 1,3-d(GpNpG) intrastrand, interstrand and protein-DNA cross-links. Because of geometric constraints, the clinically inactive *trans*-DDP isomer can not form such 1,2-intrastrand cross-links. Therefore, it is likely that the ability of cisplatin to form these particular DNA adducts accounts for to its unique anticancer activity.

Platinum-DNA adducts affect many normal biological processes in the cell, including transcription. Disruption of transcription may be the most significant contributor to the anticancer activity of cisplatin. Eukaryotic transcription is a complex process requiring not only RNA pol II but also a series of general transcription factors. These factors direct RNA pol II to the appropriate promoter sequences, facilitating transcription initiation, and promoting transcription elongation. The basal transcription factors TF IIB, TF IID, TF IIE, TF IIF, and TF IIH are required for accurate initiation of RNA pol II-catalyzed transcription from a promoter sequence.
Previous studies of the effect of cisplatin on transcription were performed either in vivo or in vitro by using pure RNA polymerase or cell extracts. In vivo studies of RNA pol II transcription from a globally platinated plasmid in mammalian cells demonstrated that RNA polymerase II bypasses cis- and trans-DDP DNA adducts with efficiencies of 0-16% and 60-70%, respectively. Cisplatin can substantially reduce transcription from a mouse mammary tumor virus promoter stably incorporated into mouse cells. On the other hand, cisplatin can block synthesis of rRNA, transcribed by RNA pol I, while activity of RNA polymerase II continued to be detected throughout the nucleus of HeLa cells. These seemingly contradictory data could result from the use of different detection methods or different cell lines.

Results from studies in vitro also seem very controversial. Pure phage SP6, T3, T7 and E.Coli. RNA polymerases, as well as wheat germ RNA pol II (no transcription factors), were investigated for their activity in the presence of cisplatin. Globally platinated DNA and site-specific cisplatin intrastrand cross-links located at d(GpG), d(ApG) or d(GpTpG) sequences were used as transcription templates. Polymerases were blocked by cisplatin adducts on the transcribed strand, but not on the non-transcribed strand. Moreover, the transcription by these polymerases was blocked more efficiently on cisplatin-treated DNA than on trans-DDP-treated DNA.

These previous in vitro studies employing pure polymerases may not accurately represent the conditions in mammalian eukaryotic cells. Eukaryotic
transcription is a highly complex process requiring not only RNA pol II, but also a series of general transcription factors. These transcription factors direct RNA pol II to the appropriate promoter sequences, to facilitate initiation and to promote elongation.

Recent experiments revealed that RNA pol II stalls at a 1,3-d(GpTpG) cisplatin adduct but not at a 1,2-d(GpG) cross-link. This work employed using extracts from *Xeroderma Pigmentosum* complementation group F GM8437 fibroblast cells. The mechanism of bypass is still unclear, and the results seem in conflict with each other. In order to reconcile these conflicting data and to investigate the effect of cisplatin on transcription elongation, a 296-bp probe containing a single, site-specific 1,2-d(GpG) intrastrand cisplatin cross-link either on the transcribed or the non-transcribed strand was synthesized as the substrate for in vitro transcription experiments using either recombinant transcription system or HeLa nuclear extracts.

**Materials and Methods**

T4 polynucleotide kinase (PNK), T4 DNA polymerase, T7 DNA polymerase, T4 DNA ligase, NTPs, dNTPs, NEB buffer 2, NEB buffer 3 and T4 PNK buffer were purchased from New England BioLabs. The T4 gene 32 protein was purchased from Boehringer Mannheim. RNA Pol II and general factors were kindly provided from Prof. D. Reinberg at UMDNJ. HeLa nuclear extracts were obtained from Prof. P.A. Sharp at MIT.
γ-[^32]P-ATP (10 mCi/mL, 6000 Ci/mmol) and α-[^32]P-UTP (40 mCi/mL, 6000Ci/mmol) were obtained from Perkin-Elmer Life Science. A low mass DNA ladder was purchased from Invitrogen Life Technologies. Phosphoramidites and chemicals for DNA synthesis were obtained from Glen Research. Agarose, LB agar, and a phenol/CHCl₃/isoamyl alcohol mixture were purchased from GibcoBRL. SequaGel diluent and concentrate solutions were purchased from National Diagnostics. The ion exchange (IE) high performance liquid chromatography (HPLC) column (Dionex DNApac PA100) was purchased from Dionex and Quick Sephadex G-25 Spin columns from Roche. Other chemicals were obtained from Sigma or Mallinckrodt.

**Synthesis and Purification of DNA Fragments.** The DNA fragments (for sequences, see Appendix 7.1) were synthesized and purified as precursors for preparing the DNA 296mer. All oligonucleotides were synthesized on a 1 μmol scale with an Applied Biosystems DNA synthesizer (Model 392) and purified by conventional methods. The platinated oligonucleotides were prepared, purified and characterized as described previously.(19)

**Platination, Purification, and Characterization of Platinated DNA Primer 20GG.**
An aliquot of 179 μL of activated cisplatin (0.84 mM) was mixed with 100 μL of oligonucleotide (1.50 mM) in a 1:1 in 0.01 M phosphate buffer (pH 6.75). The mixture was incubated in the dark at 37 °C for 6-7 h. The platinated DNA products were purified by IE HPLC and characterized by Maxam-Gilbert
sequencing, mass spectrometry, UV-vis spectroscopy and atomic absorption spectroscopy (AAS).

*Synthesis of DNA 296-Pt by the Enzymatic Ligation Method.* The 296mers were prepared by a two-phase enzymatic ligation strategy from nine synthetic DNA fragments (Figure 7.6). In the first phase, 5'-end phosphorylated fragment TT#9 was annealed with fragment TT#1, and 5'-end phosphorylated fragment TT#2 was annealed with fragment TT#8. Then these four pieces of DNA were ligated together to form fragment I. In parallel, fragment TT#3 was annealed with 5'-end phosphorylated TT#7, and 5'-end phosphorylated fragment TT#4 was annealed with TT#5 and 5'-end phosphorylated TT#6-Pt separately. Then these five pieces of DNA were ligated together to form fragment II. The products were then purified on a 5% denaturing PAGE gel electrophoresis. The desired bands containing DNA fragments I and II were isolated and extracted following the QIAEX II PAGE gel extraction protocol. In the second phase, purified fragments I and II were 5'-phosphorylated, annealed, and ligated to form the final product 296-Pt. The product was purified on a 5% denaturing PAGE gel electrophoresis. The desired band containing the DNA 296-Pt was isolated and extracted following the QIAEX II polyacrylamide gel extraction protocol. The resulting DNA 296-Pt was ready for the in vitro transcription assay. The concentration of 296-Pt was estimated by using a low mass DNA ladder (Invitrogen Life Technologies). The low mass DNA ladder is composed of an equimolar mixture of six blunt-ended DNA fragments of 2000, 1200, 800, 200 and
100 bp. Electrophoresis of 4 μL of this DNA results in bands containing 200, 120, 80, 40, 20 and 10 ng of DNA respectively. The sample DNA concentration can be estimated by comparing its intensity to that of the similar sized band of the low mass DNA ladder.

*Site-Directed-Mutagenesis (SDM).* In order to improve the efficiency of the primer extension reaction, a longer precursor was needed. Two guanines were mutated to two cytosines. The original and mutated precursors are underlined as follows:

**Before SDM** 5' ... GATA CCG GTT CTC CTA CTA ... 3'

**After SDM** 5' ... GATA CCG GTT CTC CTA CTA ... 3'

The primers Pr36m1 (5' CTT GAC TTC GGT GAT GGG AGG AGA ACC GGT ATC TTC 3') and Pr36m2 (5' GAA GAT ACC GGT TCT CCT CCC ATC ACC GAA GTC AAG 3') for site-specific mutagenesis were designed, synthesized, and purified. A PCR reaction with the primers was carried out on the double-stranded circular DNA template pUC119-296 (constructed by Dr. C. Kneip). The mutated plasmid was transformed into *E. coli* XL1-Blue supercompetent cells. A 50 μL portion of transformed cells was plated on LB-Agar plates containing 100 μg/mL ampicillin and grown overnight at 37 °C. A well-isolated colony was picked from a subcloned plate, suspended in 5 mL of LB media containing 100 μg/mL ampicillin, and grown overnight at 37 °C with agitation to reach saturation. The saturated solution was added to 500 mL of LB
media containing 100 μg/mL ampicillin and left to grow overnight at 37 °C, while rotating at 200 rpm. Cells were harvested by centrifugation for 10 min at 6000 rpm in a Sorvall GS3 rotor. The resulting mutated plasmid DNA was isolated following the Quiagen plasmid preparation kit and submitted to the MIT Biopolymers Lab for DNA sequence analysis.

Synthesis of DNA 296-Pt by the Primer Extension Method.

The procedure for primer extension is diagrammed in Figure 7.7.

1. Plasmid Transformation. The circular plasmid pUC119-296, containing the unmodified transcription template for the 296mer sequence, was transformed into E. coli XL-1 blue cells. The unmodified plasmid DNA was isolated as described in the Quiagen plasmid preparation kit.

2. ssDNA Production. In the presence of VCS M13 helper phage, ssDNA of plasmid pUC 119-296 was produced by the transformed E. coli XL-1 blue cells. Briefly, a 5-250 mL portion of sterile LB containing 100 μg/mL ampicillin and 10 μg/mL tetracycline was pre-warmed to 37 °C. A portion of 1/100 volume of mid-log phase XL 1 Blue cells was mixed with phage VCS M13 stock solution, followed by incubation at room temperature for 5 min and addition to the pre-warmed LB. The infected culture was allowed to grow for 5-6 h at 37 °C, with rotation at 250-300 rpm. Kanamycin was added to give a final concentration of 70 μg/mL and the culture was allowed to grow for 12-16 h.

The solution of infected cells was centrifuged for 15 min at 9000 X g, 4 °C twice. The supernatant was transferred to a fresh flask and a 250 μL aliquot of
RNase free DNase I was added to give a final concentration of 10 U/mL. After incubation at 37 °C for 15 min, PEG 8000 (10 g) and NaCl (7.5 g) were added, and the solution was stirred for 1 h at room temperature, followed by centrifugation for 20 min at 12,000 X g, 4 °C. The supernatant was removed, and the bottle with the pellet was centrifuged for 5 min at 12,000 X g, 4 °C, after which any remaining supernatant was removed. The pellet was resuspended with 7 mL of 10 mM Tris-HCl and 1 mM EDTA (pH = 8.0), followed by three rounds of phenol extraction and ethanol precipitation. The resulting ssDNA was characterized by agarose/EtdBr gel electrophoresis.

3. Primer Extension from Circular ssDNA. Circular ssDNA (35 pmol) was annealed to the 5'- phosphorylated platinated primer 20GG-Pt (190 pmol) in 100 µL of 10 mM Tris-HCl (pH = 7.9), 10 mM MgCl₂ and 50 mM NaCl. Following annealing, the solution was mixed with 30 units of T7 DNA polymerase, 15 units of T4 DNA polymerase, and 1200 units of T4 ligase in the presence of 1.5 mM of each dATP, dCTP, dTTP, dGTP, 1 mM ATP, 50 µg/mL BSA, 7.5 mM DTT, 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 12.5 mM NaCl (modified from the conditions used by Debyser). The reaction was incubated at 37 °C for 2 h, after which a second aliquot containing 15 units of T4 polymerase and 1200 units of T4 ligase was added. The reaction was incubated at 37 °C for another 2 h. The resulting product was analyzed by 0.7% native agarose gel electrophoresis.

The primer extension product mixture was separated by CsCl/EtdBr gradient centrifugation. After centrifugation, the relative specific densities of
DNA species, including circular DNA and nicked DNA, gave rise to a banding pattern visualized by detecting intercalated EtdBr with 366 nm UV light. A syringe was used to puncture the centrifuge tube and remove the two visible bands. EtdBr was removed by extracting twice with isoamyl alcohol. CsCl was removed by spin dialysis with Centricon-100 cartridges. The recovered DNA sample was analyzed by agarose gel electrophoresis.

4. Sma I Digestion and Agarose Gel Purification of 296-Pt. The resulting DNA was subjected to Sma I digestion to get the 296-Pt fragment. The 296-Pt fragment was separated from the remaining DNA on a 0.7% agarose gel electrophoresis. The desired band containing 296-Pt was isolated and extracted following the QIAEX II agarose gel extraction protocol. The resulting DNA was annealed and ready for the in vitro transcription assay.

In Vitro Transcription with a Reconstituted Transcription System. The in vitro transcription assay was carried out as previous described.(21) Briefly, double-stranded DNA template (80-850 fmol) was mixed with 0.1 μL of RNA polymerase II, 2.6 pmol TBP, 2.6 fmol TF IIB, 0.22 pmol TF IIE, 0.166 pmol TF IIF, 1.5 μL of TF IIH and 12 U RNAse inhibitor in the total volume of 40 μL buffer containing 10 mM Tris-HCl (pH = 7.9), 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 mM KCl, 8 mM MgCl₂, 100 mM (NH₄)₂SO₄, 66 mM DTT, 20 mM HEPES-KOH, 10% glycerol, and 4% PEG 8000. The mixture was incubated at 30 °C for ~30-40 min, after which time 5-10 μCi α-³²P-UTP, 50 μM UTP and 0.6 mM of ATP, CTP, and GTP were added. The reaction was incubated for an
additional 40 min at 30 °C. For transcription from the G-less cassette template, RNase T1 was added after the reaction, followed by incubation at 37 °C for an additional 5 min. A 100-120 μL aliquot of stop mix, containing 1 mg/mL yeast tRNA, 10 mM EDTA, 100 mM NaOAc (pH = 5.5), 0.2% SDS and 100 μg/mL Proteinase K, was added, and the reaction was incubated at 37 °C for 30 min. The reaction was extracted once with 150 μL of phenol/CHCl₃/isoamyl alcohol (25:24:1, v/v/v) and precipitated with 500 μL of ice-cold ethanol. RNA transcripts were analyzed by a 6% denaturing urea PAGE gel.

In Vitro Transcription Assay with Nuclear Extracts. HeLa nuclear extracts (30 μg) were incubated with 80-200 fmol of DNA template at 30 °C for 30 min in a buffer containing 10 mM HEPES-KOH (pH = 7.9), 10% glycerol (v/v), 60 mM KCl, 7 mM MgCl₂, 0.1 mM EDTA, 1 mM ATP, 10 mM creatine phosphate, 12 ng/μL poly I-C, 2 ng/μL poly dG-dC and 7 mM DTT. A 1 μL U/G/CTP mixture (125:5000:5000 μM) and 10 μCi α-³²P-UTP (40 mCi/mL, 6000Ci/mmol) were added, and the reactions were then incubated at 30 °C for an additional 30 min. For transcription from a G-less cassette, RNase T1 was added after the reaction, followed by incubation at 37 °C for an additional 5 min. RNA transcripts were isolated and analyzed by urea-PAGE gel electrophoresis. The gel was vacuum-dried and exposed to a phosphorimager screen.
Results and Discussion

*Platination, Purification, and Characterization of DNA Primer 20GG.*

The oligonucleotide 20GG (5'-CCACAACCGGTATCTTCACC-3') was synthesized, platinated, and purified. The corresponding cisplatin modified oligonucleotide was named 20GG-Pt. The platination sites, underlined in the sequence, were confirmed by a Maxam-Gilbert reaction (Figure 7.8). The ratio of [Pt] / [DNA] is 1.00, confirming the presence of one Pt atom per DNA strand by determining DNA concentration with UV spectroscopy and platinum concentration with atomic absorption spectroscopy (AAS). The molecular weight determined by mass spectrometry also confirmed only one platinum atom on each DNA strand.

*Synthesis of DNA 296-Pt by the Enzymatic Ligation Method.*

The DNA 296-Pt was synthesized and purified by a two-phase ligation strategy. The products were separated by 5% denaturing polyacrylamide gel electrophoresis (Fig. 7.9). The final DNA 296-Pt was annealed and ready for the transcription assay. The concentration of 296-Pt was estimated to be 50 mg/L and 5 mg/L for 296mer and 296-Pt, respectively, by using agarose gel electrophoresis (Figure 7.10). The total yield was about 1-5 %.

*Site-Directed-Mutagenesis.*

In order to make the site-specifically platinated precursor longer, thereby improving the efficiency of the primer extension reaction, site-directed-
mutagenesis was carried out to replace G with C in the DNA non-transcribed strand. The success of the site-directed-mutagenesis reaction was confirmed by the DNA sequencing analysis.

*Synthesis of DNA 296-Pt by the Primer Extension Method.*

1. ssDNA Production and Purification. A stock of helper phage VCS M13 was thawed and plated with *E. Coli* XL1-Blue cell. Phage were propagated from one well-isolated phage plaque and then harvested and titered. A titer of $5 \times 10^{12}$ pfu/mL was prepared and used for subsequent ssDNA production.

Intensive investigations were carried out to optimize the conditions for ssDNA production. A total of 56 different multiplicities of infection (MOI) combined with different incubation times of ssDNA production were carried out and the corresponding products were analyzed on a 0.7% native agarose gel. Based on those 56 reactions (data not shown), the best MOI was around 100, and the best incubation time is 10-12 h. In the optimized procedure for ssDNA production, a portion of 5-250 mL sterile LB containing 100 μg/mL ampicillin and 10 μg/mL tetracycline was pre-warmed to 37 °C. A portion of 1/100 volume of mid-log phase XL1-Blue cells was mixed with phage VCS M13 stock solution (MOI=100), incubated for 5 min at room temperature, and added to the pre-warmed LB. The infected culture was grown for 1 h at 37 °C with rotation at 250-300 rpm. Kanamycin was then added to give a final concentration of 70 μg/mL and the culture was allowed to grow for another 10-12 h. The rest of procedure
followed the established method. (22) Further agarose gel purification was performed followed by extraction of the desired band from the gel. The resulting ssDNA was characterized by agarose/EtdBr gel electrophoresis (Fig. 7.11).

2. Primer Extension Reaction. The 5’ end of the platinated precursor 20GG-Pt was phosphorylated with T4 PNK. The resulting phosphorylated DNA was annealed with ssDNA.

Intensive investigations were carried out in order to improve the product yield of the primer extension step. For the annealing efficiency, one of the most important steps, several precursors with different lengths were designed, and the corresponding site-directed mutagenesis of the plasmid was carried out. The length of the precursor was increased from 12, 16, 18, to the final 20GG-Pt. Several different polymerases including T4 and T7 DNA polymerase and correspondingly different reaction buffers were used. In addition, different incubation times and temperatures were tried. In the final optimized conditions, circular ssDNA (35 pmol) was annealed to 5’- phosphorylated platinated primer (190 pmol) in 100 µL of 10 mM Tris HCl (pH = 7.9), 10 mM MgCl$_2$ and 50 mM NaCl. Following annealing, the solution was mixed with 30 units of T7 DNA polymerase, 15 units of T4 DNA polymerase and 1200 units of T4 ligase in the presence of 1.5 mM each dATP, dCTP, dTTP, dGTP, 1 mM ATP, 50 µg/mL BSA, 7.5 mM DTT, 40 mM Tris HCl (pH 7.5), 10 mM MgCl$_2$, 12.5 mM NaCl (modified from the condition used by Debyser). (20,22) The reaction was incubated at 37 °C for 2 h, after which a second aliquot of 15 units of T4 DNA polymerase and 1200
units of T4 DNA ligase were added. The reaction was incubated at 37 °C for another 2 h. The resulting product was analyzed on a 0.7% native agarose gel (Fig. 7.12). The primer extension product mixture was separated by CsCl/EtdBr gradient centrifugation. The recovered platinated supercoiled plasmid DNA, then digested with Sma I DNA was separated by agarose gel electrophoresis, and the desired band corresponding to 296 bp was extracted.

In Vitro Transcription Assay.

The ds 296mer with a single site-specific 1,2-d(GpG) intrastrand platinated lesion was used in an in vitro transcription assay by the completely reconstituted system containing RNA pol II, TF IIH purified from HeLa cells, and recombinant TBP, TF IIB; TF IIE, TF IIF purified from E. coli. The G-less cassette template was run as a control of transcription in addition to a ds 296mer containing no platinum lesion.

Transcription from the unplatinated template produced an RNA product corresponding to a full-length transcript (around 182 nt), whereas transcription from the platinated template, produced a truncated product of approximately 121 nt (Figure 7.13). Such a difference in transcript length matches the location of platinum modification site, indicating that the 1,2-d(GpG) platinum-DNA lesion blocks RNA pol II transcription efficiently. A similar difference in transcript pattern was also observed by comparing results from unplatinated 296mer (Fig. 7.14, lane2) and platinated 296mer (Fig. 7.14, lane 3) by incubating with HeLa nuclear extracts. The RNA transcripts produced from an unplatinated template
were about 60 nt longer than the RNA transcripts from the platinated template. However, the absolute lengths of the RNA transcripts are longer than expected. It is possible that RNA Pol II binds and starts transcription from the blunt-end or near the end of linear DNA instead of the normal starting site (promoter-independent or non-specific transcription), thereby producing a longer RNA transcript. In fact, the RNA transcripts from circular DNA template were abolished when the nuclear extract was pretreated with TFIIB antibody, while RNA transcripts from linear DNA templates were only slight affected, indicating that most of the transcription from linear DNA is TFIIB independent. (Figure 8.9, Chapter 8)

The difference in RNA transcription indicates that the presence of 1,2-d(GpG) intrastrand cross-link on the template strand abolishes the full-length transcripts, producing only truncated transcripts. These results are consistent with recent reports using T7 RNA polymerase and RNA pol II from rat liver. (17,23)

A parallel in vitro transcription experiment was performed with a DNA template containing a site-specifically platinated coding (non-transcribed) strand (data not shown). In this case, the full-length transcripts are reduced, but not totally abolished. Similar to our reports, Tornaletti et al. observed that 1,2-d(GpG) cisplatin-modification in the transcribed DNA strand constitutes a strong physical barrier to RNA pol II, whereas 1,2-d(GpG) cisplatin-modification in the non-transcribed DNA strand does not.(23) We did not observe the almost 100%
RNA Pol II bypass of 1,2-d(GpG) adduct as did Culliane, however. This apparent conflict could be due to differences in the preparation of the modified DNA template, HeLa nuclear extracts or conditions of the in vitro transcription assay.

In addition, the degradation of the DNA template during incubation with the transcription system is observed, presumably by exonuclease activity. Such a degradation problem compelled us to develop the dumbbell DNA template described in Chapter 8.

In summary, here we show that cisplatin-DNA adducts blocks RNA Pol II transcription differentially based on strand location. A 1,2-d(GpG) cisplatin-DNA cross-link on the transcribed strand blocks RNA pol II more efficiently compared to the same adduct on the non-transcribed strand. Such RNA polymerase blockage by platinum-DNA adducts plays an important role in the cellular processing of cisplatin. Arrested RNA polymerase functions as a damage recognition factor and triggers several signaling transduction pathways, facilitating transcription-coupled repair or apoptosis. The detailed mechanisms of such signaling transduction pathways, such as the nature of the signal during RNA Pol II blockage at cisplatin-DNA adducts and the propagation of this signal to downstream processes, deserve further investigation. Such information will provide valuable clues about how to regulate such processes and rationally design of more efficient platinum-based drugs and therapeutic strategies.
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References


Figure 7.6. Two-phase enzymatic ligation strategy for synthesizing a platinated 296mer.
Figure 7.7. Procedure for synthesizing a platinated DNA 296mer by the primer extension method.
Figure 7.8. Maxam-Gilbert sequencing results for 20GG-Pt. Lane 1 (from left), G reaction of 20GG-Pt; lane 2, G reaction of 20GG; lane 3, G+A reaction of 20GG-Pt; lane 4, G+A reaction of 20GG.
Figure 7.9. Denaturing PAGE of the two-phase ligation reaction product and purified 296-Pt. A. Denaturing PAGE of the two-phase ligation reaction product. Lane 1, 100-bp DNA markers; lane 2, the ligation reaction in which the top strand is labeled; Lane 3, the ligation reaction with labeled bottom strand. The band near 300bp is 296-Pt (arrow). B. Denaturing PAGE of purified 296-Pt and Fragments I and II. Lanes 1 and 2, the purified fragment II, labeled on the top strand (TT3) and on the bottom strand (TT6), respectively; lanes 3 and 4, the purified fragment I, with labels on TT1 and TT9, respectively; lanes 5 and 6, the purified 296-Pt, labeled on the top strand or on the bottom strand; lane 7: phase 2 reaction product.
Figure 7.10. Concentration of 296mer is estimated by using the low mass DNA ladder. Lane M, Hi-Lo DNA marker; lanes 1-3, low mass DNA ladder, 2 μL, 4 μL and 8 μL, respectively; lane 4: 2 μL 296mer.

Figure 7.11. Agarose gel electrophoresis analysis of purified single-stranded DNA product. Lane 1 (from left), ssDNA; lane 2, dsDNA as control; lane 3, Hi-Lo DNA marker.
Figure 7.12. Agarose gel electrophoresis analysis of DNA primer extension products. Lane 1, ssDNA product; lane 2, dsDNA as control; lanes 3-4, T4, 7 primer extension. The supercoiled, nicked and gaped DNA products from primer extension reaction are indicated (arrows).
Figure 7.13. In vitro transcription assay with a cisplatin-modified template. Lane M, 100-bp ladder; lane 1, transcription products from 296-Pt; lanes 2 and 3, transcription products from 296mer; lane 4, transcription products from G-less cassette DNA.
Figure 7.14. In vitro transcription assay with a cisplatin-modified template. Lane 1, 100-bp ladder; lanes 2, transcription from 296mer; lane 3, transcription from 296-Pt.
Appendix 7.1

Sequence of 296mer (5' → 3')

Coding Strand (Non-Transcribed Strand):
GGGCATCCAAGTACTAACCAGCGGAGGACTGTCTCCGAGCGGAGGAC
TGTCCTCCGAGCGGAGGAGATTATGTGGCCTGGGCTATAAAAGGGGTGG
GGGCAGCTTCGCTTCTCACCTCTCTTCTGCTCTTTAATTTCAAGGAGTT
GGGCGCGTTCGTCCTCACTCTTCCTGCTCTTACTTTCCAGGGATTCTAA
GCCGAAGAGCTACAATCTGGACCTAGCTTTAAAATACCTTAACTTTCATC
AAGCAAGAGCGCTACGACCATACCATGGTGAAGATACCGGTTGTGGTC
CCGATCACCGAAGTCAAGCAGCATAGGGCTCGGTTAGTACTTGGATGCC
ATCACCAGAAGTCAGCAGCATAGGGCTCGGTTAGTACTTGGATGCC

Template Strand (Transcribed Strand):
GGGCATCCAAGTACTAACCAGCGGAGGACTGTCTCCGAGCGGAGGAC
ACCACAACCAGGATCTCTGAGGCTACCCGCTACCTACTGCTCAGGG
AAAGTTAAGGTTATTTAAGGTCGAGGATGGATGACGTCATCGGCTTAA
AAATCCCTGGAAAGTAAAGGCGGAAAGAGAGGAGTCGGACGACCGCAC
CCACCCCCCTTTTATAGGGCCAGGACACATAACTGAGCTCGCCGAGGAGA
TCCTCCGCTCGGAGGACAGTCAGTCCTCCGCTCGGTTAGTACTTGGATGCC

DNA fragments to synthesize DNA 296-Pt/TS

Fragment TT#1 (67 mer)
GGGCATCCAAGTACTAACCAGCGGAGGACTGTCTCCGAGCGGAGGAC
TGTCCTCCGAGCGGAGGAC

Fragment TT#2 (87 mer)
ATTATGGGCCTGGCATATAAAGGGGTGGGCGGCGGCTTCGCTCCTACT
CTCTTCCTGCTCTTTACCTTCAGGGATTCTAAGGCG

Fragment TT#3 (62 mer)
ATGACGTCATAACACCTGACCGGTTTAATACCTTACCTACGCAAGGCG

Fragment TT#4 (80 mer)
CCATACCAGTGAGATACCGGTTGAGTCCGCACTACCGAAAGTCAGAC
AGCATAGGGCTCGGTTAGTACTTGGATGCC

Fragment TT#5 (51 mer)
GGGCATCCAAGTACTAACCAGGCCCCATATGCTGCTTGACCTCGGTGATCGG
A

Fragment TT#6 (20GG-Pt)
CCACAACCGGTATCTTCACC

Fragment TT#7 (79 mer)
ATGGTATGGTCGTAGGCTCTTGCTTGATGAAAGTTAAGGTATTTAAAGGG
TCAGGGATGTTATGACGTCATCGGCTTAG

Fragment TT#8 (87 mer)
AAATCCCTGGAAAGTAAGAGCAGGAAGGAGGAGAGTGGAGGACGAACGC
CCACACACCTTTATAGCCAGCCACATACATCGCCCTCG

Fragment TT#9 (59 mer)
CCGGAGGACAGTCCTCCGCTCGGAGGACAGTCCTCCGCTCGGTAGTACT
TGGATGCCC

DNA fragments to synthesize DNA 296-Pt/CS
49 mer
CGATCACCGAAGTCAAGCAGCATAGGGCTCGGTTAGTACTTTGGATGCCC

72 mer
GGTCGTAGGCTCTTGCTTGATGAAAGTTAAGGTATTTAAAGGGTCAGGGA
TGTTATGACGTCATCGGCTTAG

73 mer
ATGACGTCATAACATCCCTGACCCTTTAATACCTTAACCTCATCAAGC
AAGAGCCTACGACCATAACCATGA

78 mer
GGGCATCAAAGTACTAACCAGGCCCTATGCTGCTTGACCTCGGTGATCGG
GTGAAGATACCGGTTGTGGTCATGGTAT

Note: The other 5 pieces are same as fragments 1, 2, 6-Pt 8, 9 of DNA 296-Pt/TS.
Chapter 8 Synthesis and Characterization of Platinated Dumbbell DNA for In Vitro Transcription and Repair Studies
Introduction

Cisplatin, a potent anticancer drug, has been used to treat several types of cancers for over 25 years. Once inside cells, cisplatin forms covalent DNA adducts, mostly 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpTpG) intrastrand crosslinks. Several cellular responses, including transcription inhibition, DNA repair, cell cycle arrest, and apoptosis were induced by cisplatin-DNA adducts.(1-5)

Circular, dumbbell-shaped DNA consists of two hairpin loops at each end and a central stem DNA duplex.(6,7) Dumbbell DNA has been proposed as an intermediate for the in vivo formation of inverted dimers (giant palindromes) during gene amplification in eukaryotic systems. It has been suggested to play important roles in genetic recombination and regulation in vivo.(8,9)

The structure of dumbbell DNA brings unique advantages for several applications. Compared to linear DNAs, dumbbell DNAs are highly resistant to exonucleolytic degradation due to the absence of free termini.(10-12) In addition, the dumbbell DNAs are more easily taken up by cells than linear DNA.(8) Therefore, dumbbell DNAs have been used as minimal-sized gene-transfer vectors in gene therapy or siRNA expression vectors.(13-17) In addition, dumbbell DNA can sequester specific DNA-binding proteins, such as transcription factors.(7,15,18-22)

Site-specifically platinated double-stranded DNA probes have been prepared as valuable templates for in vitro studies of cellular processing of cisplatin, such as protein-DNA interaction, transcription, and repair.(23-25) However, the blunt-ends
of double-stranded probes promote non-specific transcription initiation, protein-binding, and exonuclease degradation. Here we report the synthesis and characterization of site-specifically platinated dumbbell DNA probes from a pair of hairpins with 3' overhangs and three or four middle fragments using enzymatic ligation. The formation of dumbbell DNA probes was confirmed by exonuclease and restriction enzyme digestion assays. The dumbbell DNA probes were then used as templates for in vitro nucleotide excision repair and transcription.

**Experimental Section**

*Synthesis and Characterization of the Dumbbell DNA probe.*

*Synthesis of Site-Specifically Cisplatin-Modified Dumbbell DNA Probes.* The site-specifically cisplatin-modified and unmodified dumbbell DNA were synthesized by ligating five or six fragments of oligonucleotides using T4 DNA ligase as described elsewhere.(25) Schemes for the synthesis of the dumbbells are shown in Figure 8.1. Oligonucleotide fragments for each DNA probe are listed in Appendix 8.1. All fragments were synthesized on an Applied Biosystems DNA synthesizer (Model 392) and purified by PAGE and IE-HPLC. The platinated probes were same as those used in Chapter 2. The ligation products of site-specifically cisplatin-modified dumbbell DNA probes were separated on an 8% denaturing PAGE gel and eluted with TE buffer over night.

*Characterization of Dumbbell DNA Product by Restriction Enzyme Digestion.* In a typical reaction, the dumbbell probe was synthesized and purified as described above; one
fragment (20-mer) was 5'-32P labeled. The internal 32P-labeled dumbbell DNA probe (1 fmol) was incubated with the restriction enzyme (EcoR I, BamH I, Hha I) at 37 °C for 30 min. The reaction was quenched with SDS and proteinase K and then analyzed on an 8% denaturing and a 5% non-denaturing PAGE gel.

*T7 Exonuclease Digestion.* The internally 32P-labeled dumbbell DNA probes or linear DNA probes (1 fmol) were incubated with 5 U of T7 exonuclease enzyme in 50 mM KOAc, 20 mM Tris-HOAc (pH = 7.9), and 1 mM DTT at 25 °C for 30 min. The reactions were then treated with 10% SDS and proteinase K and analyzed on an 8% denaturing PAGE gel.

*Excision Assay of Dumbbell DNA.*

The dumbbell d(GpG)-Pt and d(GpTpG)-Pt probes were used as substrates for the nucleotide excision repair assay as described.(25) The AA8 CHO cell-free extracts were added into the mixture and incubated at 30 °C for 1.5 h. The reaction products were then treated with proteinase K and SDS at 30 °C for 15 min, extracted with phenol, and precipitated by ethanol. The repair products were analyzed on an 8% denaturing gel.

*Reconstitution of Site-Specifically Cisplatin-Modified Nucleosome.*

The internally 32P-labeled dumbbell DNA probes were assembled into nucleosomes with a reconstituted histone octamer as described.(25) Briefly, DNA substrate (1 pmol) was incubated for 15 min at 37 °C with the core histones in a 1:1
molar ratio in a final volume of 10 μL solution containing 1 μg of BSA and 2 M NaCl. The reaction mixture was serially diluted with portions of 50 mM HEPES (pH = 7.5), 1 mM EDTA, 5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) over a period of 2.5 h incubating at 30 °C. The resulting solution were mixed with a 100 μL of 10 mM Tris HCl (pH = 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 5 mM DTT, 0.5 mM PMSF, 20% glycerol, and 100 μg/mL of BSA and then incubated for 15 min at 30 °C.

**In Vitro Transcription Assay with DNA Template Containing a G-less Cassette.**

Transcription reactions (25 μL) were performed essentially as described using 10 μCi α-32P-CTP as the radiolabel, together with a C/G/UTP mix (25:200:200 μM).(26) The DNA templates used was a BamH I linearized plasmid pG5MLP-G380 (containing a 380 nucleotide G-less cassette and a major late promoter) and a circular (supercoiled) plasmid pHIV/TAR-G100 containing a 100 nucleotide G-less cassette and a mutant HIV promoter. Antibodies to TFIIB or c-myc were added where indicated. RNA transcripts were treated with RNase T1 and G-less transcripts analyzed on a 6 % urea-polyacrylamide gel electrophoresis.

**Assessment of Transcription from Dumbbell DNA using RNase Protection Assay.**

Transcription reactions were performed as described above, except that unlabelled C/G/UTP (200 μM each) was used in the reactions. RNA transcripts were isolated essentially as described,(27) and analyzed using the RNase protection
assay (RPA) kit from Ambion. The radiolabelled probe for the RPA assay was generated by the polymerase chain reaction (PCR) using the dumb-bell DNA as a template, 10 µCi α-\(^{32}\)P-dATP as the radiolabel. As a negative control, yeast RNA was included in an RPA reaction in the absence of a transcript.

**Results and Discussion**

*Synthesis and Characterization of Dumbbell DNA Probes*

All oligonucleotide fragments were purified by PAGE gels. The purification and characterization of platinated oligonucleotides DNA 20GG-Pt and 20GTG-Pt were performed as reported.(25)

To test the ligation efficiency of each fragment of dumbbell DNA, a 5'-\(^{32}\)P-labeled 65-mer was mixed with either the 89-mer (Figure 8.2, lane 2); the 89-mer and 20-mer (Figure 8.2, lane 3); the 89-mer, 20-mer, and 88-mer (Figure 8.2, lane 4); or the 89-mer, 20-mer, 90-mer and 88-mer (Figure 8.2, lane 5) in the ligation buffer. As indicated in Figure 8.2, the bands corresponding to the predicted ligation products were observed in the each lane. Lane 1 shows the presence of the starting 65-mer and the ligation product 154-mer (65+89). Bands corresponding to starting 65-mer, the ligation products 85-mer (65+20), 154-mer and 174-mer (65+89+20) were observed. Lane 3 shows the presence of the starting 65-mer, as well as the ligation products 85-mer, 154-mer, 174-mer, and 262-mer (65+89+20+88). In lane 4, the band
corresponding to full-length ligation product was observed. The structure of dumbbell DNA allows it to migrate more rapidly than linear DNA of similar length.

Due to the low yield of the 90-mer, a six-fragment strategy was then applied to synthesize greater amounts of dumbbell DNA (Figure 8.1). The 90-mer was replaced by a 68-mer and a 22-mer and six parallel ligation experiments were performed to identify the band corresponding to the dumbbell product. In each ligation experiment, one of six fragments was labeled by $\gamma$-32P-ATP. The six parallel reactions were analyzed on a denaturing PAGE gel (Figure 8.3). The band containing dumbbell, if any, should be observed in all six lanes. The top band observed in each lane was cut out and eluted for further characterization.

A restriction enzyme assay was used to identify further the dumbbell product. Restriction sites of BamH I, Hha I, and EcoR I are located within dumbbell as shown in Figure 8.4. A missing fragment would cause the product to lose restriction digestion site of at least one enzyme. Figure 8.5 show that the ligation product was digested by all three enzymes. This result indicates that the ligation product contains all six fragments. Addition of T7 exonuclease did not degrade the ligation product after 30 min, whereas linear DNA was digested (Figure 8.6). The rapid mobility band in lane 2 is the partial digestion product in which T7 exonuclease is blocked by the cisplatin lesion.

Taken together, these results indicate successful synthesis of the dumbbell DNA probe. It could be used as a probe to study transcription-coupled repair and global-genome nucleotide excision repair as well utilized in other applications.
Nucleotide Excision Repair from Dumbbell Probes. The internally $^{32}$P-labeled dumbbell GG-Pt and GTG-Pt (352 nt) were compared with the linear 146GG-Pt and 146GTG-Pt for efficiency of NER. As shown in Figure 8.7, the dumbbell DNA probes exhibit a higher repair signals (about 2 fold) than the linear DNA probes. In addition, the similar stimulation results were observed when comparing the excision of dumbbell probes and their linear counter parts generated by double restriction enzyme digestion (data not shown).

The structural or topological change of DNA induced by the formation of dumbbell ends might account for such stimulated of excision. However, it may not be the main factor. Although studies of small dumbbell DNA (<10 nt) report that the two hairpin loops and formation of dumbbell changes the DNA structure,(28,29) very little change was observed in longer dumbbell DNA due to loop ends on the DNA (>16 bp)(30) indicating the effect of hairpin loops did not propagate over a long range.

Alternatively, some protein factors, such as end processing proteins, interact differentially with dumbbell DNA and linear DNA, thus modulating excision repair differently. For example, the Ku70/80 protein complex can bind to the ends of DNA and inhibit the binding of nucleotide excision repair proteins such as XPA and p62-TFIH on linear damaged DNA in vitro.(31-34) Studies of the mode of Ku interaction with DNA revealed that the addition of two hairpin loops to the same 25-bp linear substrates change the Kd values from 3.8 nM (linear) to 11.4 nM (dumbbell).(35) The
contribution of Ku70/80 on such a stimulation in repair from dumbbell deserves further investigation.

Nucleosome Assembly of Dumbbell DNA. To test whether the formation of dumbbell DNA affects nucleosome reconstitution, the dumbbell DNA was assembled with histone octamer to form a nucleosome. As shown in Figure 8.8, a significant amount of DNA was incorporated into nucleosomes. The presence of dumbbell ends does not inhibit the formation of a nucleosome. This is the first synthesis of a dumbbell nucleosome. This dumbbell nucleosome could be used for in vitro transcription template or repair template to probe cisplatin processing at nucleosomal level.

Cisplatin Blocks the Transcription from Dumbbell Probes. The cisplatin-modified and unmodified dumbbell DNAs were synthesized to investigate the effect of cisplatin on transcription elongation. As shown in Figure 8.9, the band corresponding to the full length RNA transcript (Figure 8.9a, lane 4, 8) had disappeared in cisplatin-modified DNA (Figure 8.9a, lane 11), indicating that RNA transcript is blocked by cisplatin-DNA adducts.

TFIIB Dependent Transcription from Dumbbell Probes. One problem in using linear probes as transcription templates is that a significant amount of RNA transcripts form in TFIIB-independent manner. RNA polymerase can bind to the blunt ends of DNA and start transcription without the help of TFIIB. As shown in Figure 8.9 b, when HeLa nuclear extracts containing circular or linear DNA were pretreated with TFIIB antibody, the RNA transcripts were abolished or slightly
affected, respectively. When the cell extracts were pretreated with anti-TFIIB, the RNA transcripts were significantly reduced. This result indicates that the most transcription from the dumbbell is TFIIB dependent.

We have synthesized and characterized the site-specifically cisplatin-modified dumbbell DNA probes. The unique dumbbell structure makes it a promising candidate or substrate for a variety studies in vitro or in vivo. For example, the site-specifically platinated dumbbell is a good template for transcription and repair. In addition, a photo-crosslinking study using dumbbell DNA as probe has identified novel proteins that bind to platinum-DNA adducts. Furthermore, platinated dumbbell DNA could be transfected into cells to regulate cellular processes by reducing the availability of proteins, such as HMG family proteins or TBP in cells.

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Five-Fragment Strategy

![Diagram of Five-Fragment Strategy]

Six-Fragment Strategy

![Diagram of Six-Fragment Strategy]

Figure 8.1. Strategies for the synthesis of site-specifically platinated dumbbell DNA probes. Five (top) or six (bottom) synthetic oligonucleotides were annealed and ligated as shown. Sequences of the individual strands are listed in Appendix 8.1.
Figure 8.2. Denaturing PAGE gel analysis of dumbbell ligation products. Lane 1, 100 bp DNA ladder; lane 2, 5'-32P labeled 65mer ligated with the 89mer; lane 3, 5'-32P labeled 65mer ligated with the 89mer and 20mer; lane 4, 5'-32P labeled 65mer ligated with the 89mer, 20mer, and 90mer; lane 5, 5'-32P labeled 65mer ligated with the 89mer, 20mer, 90mer, and 88mer.
Figure 8.3. Denaturing PAGE gel analysis of dumbbell ligation products (six-fragment strategy). Lane 1, 10 bp DNA ladder; lane 2, 100 bp DNA ladder; lane 3, 5'-32P labeled 89mer ligated with the 88mer, 68mer, 65mer, 22mer, and 20mer; lane 4, 5'-32P labeled 88mer ligated with the 89mer, 68mer, 65mer, 22mer, and 20mer; lane 5, 5'-32P labeled 68mer ligated with the 89mer, 88mer, 65mer, 22mer, and 20mer; lane 6, 5'-32P labeled 65mer ligated with the 89mer, 88mer, 68mer, 22mer, and 20mer; lane 7, 5'-32P labeled 22mer ligated with the 89mer, 88mer, 68mer, 65mer, and 20mer; lane 8, 5'-32P labeled 20mer ligated with the 89mer, 88mer, 68mer, 65mer, and 22mer; lane 9, purified dumbbell DNA ligation product (arrow).
Figure 8.4. Digestion sites of restriction enzymes on the dumbbell DNA. The restriction locations are indicated.
Figure 8.5. Non-denaturing gel of restriction enzyme digestion of dumbbell DNA. Lane 1, 100-bp ladder; lane 2, untreated dumbbell DNA GTG; lane 3, EcoR I digestion of dumbbell DNA GTG; lane 4, BamH I digestion of dumbbell DNA GTG; lane 5, Hha I digestion of dumbbell DNA GTG; lane 6, untreated cisplatin-modified dumbbell DNA GTG-Pt; lane 7, EcoR I digestion of cisplatin-modified dumbbell DNA GTG-Pt; lane 8, BamH I digestion of cisplatin-modified dumbbell DNA GTG-Pt; lane 9, Hha I digestion of cisplatin-modified dumbbell DNA GTG-Pt.
Figure 8.6. Denaturing gel of T7 exonuclease digestion of dumbbell DNA. The internally labeled linear (195GG-Pt) and dumbbell DNA probes (db-GTG and db-GTG-Pt) were incubated with 5 U of T7 exonuclease enzyme (lanes 2, 4, and 6, respectively), at 25 °C for 30 min. Lanes 1, 3, and 5 are untreated 195GG-Pt, db-GTG, and db-GTG-Pt, respectively.
Figure 8.7. Denaturing PAGE gel of repair assays for dumbbell DNA. Lane 1, NER assay of dumbbell GG-Pt; lane 2, NER assay of dumbbell GTG-Pt; lane 3, NER assay of 146 GTG-Pt; lane 4, NER assay of 146 GG-Pt.
Figure 8.8. Reconstitution of dumbbell into nucleosome. Lane 1, Nucleosome 199GTG; lane 2, product from dumbbell nucleosome assembly reaction; lane 3, free dumbbell DNA.
Figure 8.9. Effect of anti-TFIIB on transcription from dumbbell DNA.
Appendix 8.1
Dumbbell GG and GTG sequences: (5’ → 3’)

Fragment 89mer
GCGGTTCGTCCTCCTCCTCATCTCTCTGACTAGCTGACTTTGGGTGAAGATACCGGTTGGCGGAGTAGTCTCCGAATAGT

Fragment 65mer
GAGTCGTATTACCGAATTTGCGTAATACGACTGACTATTCGGACTAA

Fragment 88mer
CAAATCGGAGTCAGAGAGTTGCAGTAGGAAGAGTAGGAGGACGACGGCGCCTCCACCC
CTTCTATAGGCCCTCTTCAGGATCCGT

Fragment 68mer
ATTCTATAGTGGTAGCCTAATCGCCATCAGGGAGTTTAGGAGCAGACATCATAGAATACGGGGTAAG

Fragment 22mer
5’-GGGGCTATAAAAGGGGGTGGGG-3’

Fragment 20mer GG
5’-a atcct cctGG ttttt ccac-3’

Fragment 20mer GTG
5’-a atcct ccGTG ttttt ccac-3’

Fragment 89mer GG
GCGGTTCGTCCTCCTCCTCATCTCTCTGACTAGCTGACTTTGGGTGAAGATACCGGTTGGCGGAGTAGTCTCCGAATAGT

Fragment 89mer GTG
GCGGTTCGTCCTCCTCCTCATCTCTCTGACTAGCTGACTTTGGGTGAAGATACCGGTTGGCGGAGTAGTCTCCGAATAGT
Appendix A1 The Interaction of Human SWI/SNF with Platinated Nucleosomes and Its Effect on NER
Introduction

Cisplatin has been widely used as a potent anticancer drug for many years to treat several kinds of cancer, including testicular, ovarian, head and neck, and non-small cell lung cancers. Cisplatin forms covalent DNA adducts, primarily 1,2- or 1,3- intrastrand cross-links and a smaller number of interstrand cross-links as well as protein-DNA crosslinks. (1,2) These cisplatin-DNA adducts are responsible for triggering several cellular responses, including transcription inhibition, DNA repair, cell cycle arrest, and apoptosis. (1,3-6) Nucleotide excision repair is a major cellular defense mechanism against the toxic effects of cisplatin. The NER machinery recognizes the cisplatin lesion, unwinds the nearby DNA duplex, removes 24 to 32 nucleotides containing the cisplatin-DNA adducts, and promotes the synthesis and ligation of new DNA to fill the resulting gap. (7, 8)

In eukaryotic cells, DNA is packaged into chromatin. Nucleosomes, the fundamental unit of chromatin structure, are composed of 146 bp of DNA wrapped around a histone octamer in a shallow, left-handed helix. The histone octamer is a tripartite arrangement assembled from one (H3/H4)\textsubscript{2} tetramer and two H2A/H2B dimers. (9) Chromatin adds another level of complexity to the regulation of the DNA related processes such as gene expression, DNA
recombination, and repair. DNA that is contained within highly compacted chromatin is inaccessible to the transcription and repair machinery. Previous work from our laboratory revealed that the nucleosome significantly inhibits nucleotide excision repair in comparison with the extent of repair by mammalian cell extracts of free DNA containing the same site-specific platinum-DNA adduct in vitro. (10)

Two major classes of factors have been identified to alter DNA accessibility in chromatin. The first class regulates chromatin structure by reversible formation of covalent histone modifications.(11,12) Histone acetylation, phosphorylation, methylation and ubiquitination all have a role in the regulation of gene expression from chromatin.(13,14) In addition, the post-translational modification of histones can also modulate nucleotide excision repair from damaged chromatin in vitro.(10) By changing the chemical characteristics of the histone tails, histone-DNA interactions are reduced, or alternatively, other factors are recruited to the nucleosome, thereby altering the accessibility of the nucleosomes to the repair or transcription machinery.

The second class of factors remodel chromatin in an ATP-dependent fashion, utilizing ATP hydrolysis to change histone-DNA contacts within the nucleosome.(15, 16) These complexes can be divided into three groups on the basis of the similarities of their ATPase subunits, namely, Swi2/Snf2, Isw1, and Mi-2. All three classes play very important roles in nuclear events such as transcription, replication, recombination, and DNA repair.(16-18)
SWI/SNF, one of best characterized ATP-dependent remodeling complexes, was first identified in the yeast *Saccharomyces cerevisiae*. This 2 MDa multi-subunit complex is highly conserved in eukaryotes. The mechanism of nucleosome remodeling by SWI/SNF remains to be fully elucidated, although several potential mechanisms, such as twist defect diffusion and bulge diffusion, have been proposed.

Recently, a growing body of evidence has linked the activity of the SWI/SNF complex to DNA repair and recombination within nucleosomes. SWI/SNF enhances cleavage of the V(D)J recombination signal sequence in the mononucleosome by the RAG1/RAG2 recombinase. In addition, association of BRCA1 with SWI/SNF implies that the SWI/SNF chromatin-remodeling complex might have a role in DNA repair. Direct evidence that yeast SWI/SNF complexes enhance the excision of UV and AAF lesions from nucleosomes is available from in vitro studies. The stimulatory effect of SWI/SNF on NER depends upon the nature of DNA lesion. SWI/SNF stimulates the excision of AAF-adducts and the (6-4) photoproducts from the nucleosome, whereas it has a negligible effect on the repair of TT dimer. These differential effects of SWI/SNF on repair of UV photoproducts and the AAF adduct stimulated our interest to investigate the effect of SWI/SNF complexes on the repair of cisplatin damage within nucleosome. In this chapter, we address the effect of SWI/SNF on the repair of cisplatin-DNA adducts in synthetic mononucleosomes. A moderate stimulation by SWI/SNF on repair is reported.
Materials and Methods

Materials. Cisplatin was obtained as a gift from Engelhard. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs. Phosphoramidites and chemicals for DNA synthesis were obtained from Glen Research. γ-32P-ATP was purchased from Perkin-Elmer Inc.

Synthesis and Purification of Site-Specifically Platinated DNA Probes. The site-specifically platinated DNA duplexes were prepared by enzymatic ligation of complementary oligonucleotides as previously described.(7) Briefly, four DNA fragments and one site-specifically cisplatin-modified oligonucleotide were phosphorylated using T4 polynucleotide kinase and ATP. All the fragments were then annealed and ligated together using T4 ligase. The full length ligation products were then separated from partial ligation products by denaturing PAGE, and subsequently isolated by non-denaturing PAGE.

Proteins Preparation. Cell-free extracts were prepared from CHO AA8 cells by the method as described previously.(27) hSWI/SNF complex was prepared by Dr. H. Y. Fan as described.(28,29) The four histone proteins were expressed in E. coli BL21(DE3) cells and purified following the published procedures.(30, 31) The histone octamer was refolded, dialyzed, and then separated on a 16/60 Superdex 200 gel filtration column (Pharmacia).
Nucleosome Assembly of Site-Specifically Cisplatin-Modified Nucleosomes. The internal $^{32}$P-labeled DNA probes (146GG-Pt or 146GTG-Pt) were assembled into nucleosomes with reconstituted histone octamers as described.(10) Briefly, 1 pmol of DNA substrate was incubated with the core histones in a 1:1 molar ratio with 1 μg of BSA and 2 M NaCl in a final volume of 10 μL for 15 min at 37 °C. The reaction mixture was serially diluted with portions of 50 mM HEPES (pH 7.5), 1 mM EDTA, 5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) over a period of 2.5 h, incubating at 30 °C. The resulting solution was brought to 0.1 M in NaCl by adding 100 μL of 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 5 mM DTT, 0.5 mM PMSF, 20% glycerol, and 100 μg/mL of BSA, and incubated for 15 min at 30 °C.

Glycerol Gradient Purification. The nucleosome products were purified from free DNA by glycerol gradient centrifugation in a similar manner as described.(28) Briefly, nucleosomes were layered onto a 10-30% glycerol gradient buffer (50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 100 mM KCl, 10 or 30% glycerol) and spun at 35,000 rpm in a SW-41 rotor (Beckman Instruments) for 16 h. The fractions were collected and characterized by 5% non-denaturing gel at 4 °C. The purified nucleosomes were used for the NER assay.

DNase I Footprinting Assay. About 10 fmol of 5'-$^{32}$P labeled 146GTG or 146GTG-Pt mononucleosomes were treated with 0.2 U DNase I in 20 μL TE buffer (10 mM Tris·HCl (pH 8.0), 1 mM EDTA) at 25 °C for 1 min or 5 min. The reactions were quenched by 2 μL of 15.3 mg/mL proteinase K/ 10% (w/v) SDS
at 30 °C for 15 min, followed by phenol extraction and ethanol precipitation. The reaction products were analyzed on a 6% denaturing PAGE gel.

Excision Assay for Nucleosomes Preincubated with SWI/SNF. A portion of 0.1 nM internally labeled mononucleosome substrates was pre-incubated with 320 ng of SWI/SNF in 1.6 mM Tris HCl (pH = 7.9), 33.6 mM Hepes (pH = 7.9), 72 mM KCl, 5.44 mM MgCl₂, 2.4 mM ATP, 200 ng/μL BSA, 0.08% NP40, 4 ng/μL pUC18, 7.3% glycerol, 0.48 mM DTT, 0.56 mM EDTA, 1.6 mM β-mercaptoethanol and 0.032 mM PMSF at 30 °C for 60 min. The AA8 CHO cell-free extracts were added into the mixture and incubated at 30 °C for another 3 h. The reaction products were then treated with proteinase K (15.3 mg/mL)/SDS (10% w/v) buffer at 30 °C for 15 min, followed by phenol extraction and ethanol precipitation. The repair products were analyzed on an 8% denaturing PAGE gel.

Restriction Enzyme Characterization. To test the remodeling activity of SWI/SNF on mononucleosomes, 0.1 nM internal ³²P-labeled mononucleosome substrates were incubated with restriction enzyme (5 U Nla III) and, when needed, 80 ng SWI/SNF in excision buffer (see above) at 30 °C for various time. The reaction products were then treated with proteinase K/SDS buffer at 30 °C for 15 min, followed by phenol extraction and ethanol precipitation. The reaction products were analyzed on an 8% denaturing PAGE gel.
Results and Discussion

Characterization of Nucleosomes by Footprinting. The nucleosomes were prepared by the salt dilution method. The purified nucleosome was analyzed on a 5% non-denaturing gel (Figure A1.1). The cisplatin modification site is located at 70th nucleotide from 5' terminus of the DNA duplex. Analysis by DNase I footprinting revealed a strong 10-bp periodicity (Figure 4.5). The cleavage rate of nucleosomal DNA by DNase I is much slower than that for free DNA, indicating that the core histones protect DNA from DNase I cleavage (data not shown). (32)

Comparison of the cleavage patterns from platinated and unmodified nucleosomes reveals a difference over the region from the 30th to the 120th nucleotide from 5' terminus of the nucleosomal DNA (Figure 4.5). This result indicates that although the incorporation of cisplatin-DNA adduct does not affect the overall structure of nucleosome, there is a local distortion. A more quantitative measure of this structural change, revealed by hydroxyl radical footprinting is present in Chapter 4. (33)

Restriction Enzyme Characterization. To test the effect of SWI/SNF on the nucleosome substrate, Nla III digestion was used to monitor the remodeling activity. As shown in Figure A1.2, addition of SWI/SNF stimulates the digestion efficiency of nucleosome substrates from 20.3% to 23.4% (4 min digestion) or from 33.8% to 39.4% (20 min digestion), whereas the addition of SWI/SNF did not change the digestion efficiency of DNA substrates (93.6% versus 94.3% for 4 min digestion and 95.6% versus 96.4% for 20 min digestion). The measured
hSWI/SNF stimulation is not as dramatic as previously reported for yeast SWI/SNF.(26) There are two possible reasons for this difference. First, the excision reaction buffer is different for the two studies, and the activity of hSWI/SNF might not be optimized under these circumstances. Second, different restriction enzymes, Nla III and EcoRI, were used in the two studies. Nla III might not act as efficiently as EcoRI to digest the remodeling DNA on the nucleosome.

The Stimulatory Effect of hSWI/SNF on NER. The nucleosome substrate 146GTG-Pt was pre-incubated with SWI/SNF for 1 h at 30 °C and then incubated with cell-free extracts for another 3 h. The repair results reveal that pre-incubation with human SWI/SNF stimulates excision from the nucleosome substrate by 2.8 fold (from 0.6% to 1.7%), whereas, addition of SWI/SNF stimulates excision from free DNA by 1.7 fold (from 2.6% to 4.3%, Figure A1.3). The better stimulatory effect on DNA may due the slight differences in protein concentration between the SWI/SNF-containing and control reactions. Nevertheless, the relative stimulation (from nucleosomes vs DNAs) is 1.7 fold. The SWI/SNF complex disrupts histone-DNA contacts and thereby increases the accessibility of the platinated duplex. Previous findings revealed that SWI/SNF differentially stimulates repair of UV-damaged DNA in nucleosomes. SWI/SNF stimulates the excision of the AAF-G adduct and the (6-4) photoproduct by about 1.5 -2.0, whereas there was no effect on excision of a TT dimer.(25,26) Therefore,
the effect of SWI/SNF on repair of 1,3-d(GpTpG)-Pt is more similar to the results
for the AAF-G adduct and (6-4) photoproduct.

Previous biochemical analysis has demonstrated several different
outcomes of SWI/SNF remodeling activity in vitro, including nucleosome sliding,
the transferring of a histone octamer to another DNA molecule, and the altering
of nucleosome structure.(19) It is not clear whether these events occur by a single
or by multiple mechanisms. Several possible different mechanical forces,
including octamer sliding, DNA twisting, creating a bulge of exposed DNA, and
translocating DNA, may be involved in SWI/SNF nucleosome
remodeling.(19,20,34,35) Nevertheless, in each case, histone-DNA contacts are
weakened, thereby allowing the cisplatin-DNA lesion to be more accessible to
the repair machinery. Alternatively, local distortions of cisplatin-DNA adducts
are altered by SWI/SNF, thereby making such lesions much easier to be
recognized for repair.

Although the composition and subunit structure of human SWI/SNF and
yeast SWI/SNF are generally well-conserved, the two complexes have certain
differences. The yeast SWI/SNF complex has only one ATPase and one ARID-
containing protein, whereas human SWI/SNF has two alternative ATPase
subunits and ARID-containing subunits.(36) The DNA binding affinity of ARID
subunits in human SWI/SNF is much higher than its counterparts in yeast
complexes.(37) Moreover, human complexes also have an additional DNA-
binding component, BAF57, for which yeast complexes have no counterpart. This
HMG-domain containing subunit can bind selectively to four-way junction DNA. (38) Because of the HMG domain, which binds 1,2-d(GpG) cisplatin-DNA adducts, the interaction of BAF57 and its role in remodeling cisplatin-modified nucleosomes deserve further investigation.

**SWI/SNF and Histone Modifications.** SWI/SNF complexes cooperate with histone-modifying enzymes such as histone acetyltransferases (HAT) Gcn5 or CBP/p300 for chromatin remodeling, leading to transcriptional activation of a large number of genes. (39,40) Acetylation of histone H4 at K8 mediates recruitment of the SWI/SNF complexes. (41) The bromodomain in the ATPase subunit Swi2 of SWI/SNF, which interacts specifically with acetylated histone tails, is important for such regulation in vivo. (42) It is tempting to speculate that SWI/SNF and HAT may also act synergistically in some cases to modulate NER of platinated nucleosomes in vivo.

**The Role of SWI/SNF in Cancer.** The SWI/SNF complex is implicated in cancer since several subunits either have intrinsic tumor-suppressor activity or are required for the activity of other tumor-suppressor genes. (43,44) Moreover, specific mutation and/or loss of BRG1, an ATPase catalytic subunit of the SWI/SNF complex, has been identified in pancreatic, breast, prostate, and non-small-cell lung cancer cell lines. (45-48) The loss or mutation of subunits of SWI/SNF in tumor cell lines will alter the remodeling activity of SWI/SNF complex, the cellular repair activity of cisplatin-DNA adducts will therefore be
also affected. This differential activity of SWI/SNF in cancer vs normal cells may partially contribute to the anti-cancer activity of cisplatin.

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References


Figure A1.1. Analysis of site-specifically cisplatin-modified nucleosome on a 5% non-denaturing PAGE gel electrophoresis.
Figure A1.2. The effect of hSWI/SNF on nucleosome structure characterized by Nla III digestion. Mononucleosome substrates (A) or free DNA (B) were incubated with 5 U of Nla III and, when needed, 80 ng SWI/SNF in excision buffer at 30 °C for 4 min or 20 min. The reaction products were analyzed on an 8% denaturing PAGE gel. The digestion efficiencies are indicated. The digestion site of Nla III and platination site are shown above.(C)
Figure A1.3. The stimulatory effect of hSWI/SNF on nucleotide excision repair. Lane 1, nucleosomal 146GTG-Pt DNA; lane 2, nucleosomal 146GTG-Pt DNA in the presence of hSWI/SNF; lane 3, free 146GTG-Pt DNA; lane 4, free 146GTG-Pt DNA in the presence of hSWI/SNF.
Biographical Note

The author was born on June 12, 1975 in Beijing, P. R. China to Hua and Bao-Guo Wang. He was raised along with one young sister, Wei (Wendy) in Beijing, P. R. China, and attended the Senior High School Attached to Peking University, where his interest in chemistry and biology was inspired. After graduating in 1993, he attended college at Peking University, P. R. China, where his majored in chemistry and did undergraduate research in the purification, characterization and crystallization of a novel insect neurotoxin from the venom of the scorpion Buthus martensii Karsch. under the guidance of Prof. Genpei Li and Prof. Dacheng Wang. He earned his Bachelor of Science degree in Chemistry with the highest honor in 1998, and began his graduate work at MIT in 1999. He received Anna Fuller Fund graduate fellowship in 2002. Following graduation, he will continue his postdoctoral training in Prof. Roger Kornberg’s lab at Stanford University.
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