# Structure, Recognition, and Processing of Cisplatin−DNA Adducts

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## Contents



## I. Introduction

In the course of examining the effect of electric fields on the growth of *Escherichia coli* cells, a biological activity of platinum compounds was uncovered that led to the development of some of the most widely used anticancer drugs today.[1,2] Compounds formed by reaction of platinum from the electrodes with ammonium chloride in the buffer stopped cell division and induced filamentous growth in the bacteria. Subsequent testing of these compounds in mice revealed antitumor activity.[2,3] One of the more successful compounds, *cis*-diamminedichloroplatinum(II), or cisplatin, had been known since 1845,[4,5] but not until 1970 was its antitumor activity established.[2,3,6] Since this serendipitous discovery, cisplatin has been used to treat a variety of human malignancies.

Early clinical trials with cisplatin were promising, the one major drawback being severe renal toxicity that was ultimately overcome through hydration therapy and diuresis.[7] Cisplatin was approved by the FDA in 1978, and the cure rate for testicular cancer is now greater than 90% when tumors are promptly diagnosed.[8] Cisplatin is also used to treat other kinds of malignancies, including ovarian, cervical, head and neck, esophageal, and nonsmall cell lung cancer.[9]

The cisplatin treatment regimen generally involves a series of intravenous injections administered every 3−4 weeks at a dose of 50−120 mg/m$^2$.[9] Despite the great success at treating certain kinds of cancer, the drug does have some limitations. There are several side effects, and both intrinsic and acquired resistance limit the organotropic profile of the drug.

Over the years, various platinum complexes, some of which are shown in Figure 1, have been studied in an attempt to overcome these problems. Many of the compounds exhibiting antitumor activity have had two *cis*-amine ligands, and *trans*-diamminedichloroplatinum(II) (*trans*-DDP), the geometric isomer of cisplatin, is clinically ineffective. Carboplatin, *cis*-diammine-1,1′-cyclobutane dicarboxylate platinum-(II), has reduced toxicity but is cross-resistant with cisplatin.[10] Oxaliplatin, *trans*-L-diaminocyclohexaneoxalatoplatinum(II), displayed a lack of cross-resistance and has been used to treat colorectal cancer.[11,12] Orally active platinum(IV) compounds that would broaden treatment conditions are also in development.[13] In addition, recent work suggests that there may be some biologically active trans platinum compounds, including platinum(II) complexes with planar ligands,[14−16] platinum(II) iminoether compounds,[17,18] and *trans*-ammine(amine)platinum(IV) compounds.[19−21] Over 3000 cisplatin analogues have been tested,[22] with 28 platinum compounds, selected for some of the activities described above, having entered clinical trials.[10] Unfortunately, most of these drug candidates have encountered difficulties in the clinic, perhaps due to the fact that a specific cellular target or mechanism was not used as the basis for drug design. It is estimated that more than 10 000 compounds need to be screened in order to obtain a new, effective anticancer drug.[23] The development of new antitumor platinum compounds will not be further addressed in this review because it is covered elsewhere in this issue.

Much research has been conducted over the ∼35 years following the discovery of biological activity for cisplatin in an attempt to elucidate its mechanism

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of action. A major focus of this work has been DNA, the biological target of the drug, and examining the effects of cisplatin adduct formation on DNA−dependent cellular functions. The identification of proteins that mediate the biological response of cells to cisplatin has been a topic of current interest. The present review examines aspects of these investigations and, after discussing mechanistic issues that are not fully resolved, looks to future directions of research.

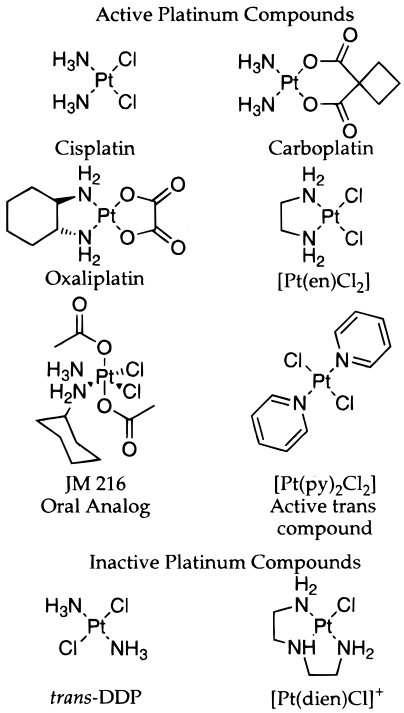


**Figure 1.** Structures of some platinum compounds investigated for biological activity.

## *II. DNA: The Biological Target of Cisplatin and Effects of Platination on Structure*

After the discovery of the anticancer properties of cisplatin, work began to investigate its mechanism of action. One of the first issues that needed to be settled was its biological target, for there are many cellular components that can react with cisplatin. Studies eventually focused on the nature of platinum binding to DNA.

### A. Potential Cellular Targets for Cisplatin

Following injection into the bloodstream, cisplatin encounters a relatively high concentration of chloride ions (100 mM) that suppresses hydrolysis and maintains the compound in a neutral state. The limiting factor for accumulating platinum in cells is its concentration, and the uptake of cisplatin is not saturable.[24−27] Cisplatin uptake does not have a pH optimum nor is it inhibited by structural analogues, such as carboplatin or *cis*-$Pd(NH_3)_2Cl_2$, suggesting that the transport is not carrier-mediated.[28] These results indicate that cisplatin enters cells by passive diffusion, although there is some evidence that uptake may in part occur by an active transport mechanism.[29] Once inside the cell, the diminished chloride ion concentration (∼20 mM) facilitates hydrolysis. The result is an activated, aquated form, *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$, which can react more readily with cellular targets.

Many cellular components including RNA, proteins, DNA, membrane phospholipids, and microfilaments that make up the cytoskeleton react with

cisplatin. The first clue for identifying the principal cellular target was the filamentous growth of the bacteria induced by cisplatin, a phenomenon characteristic of DNA−damaging agents such as UV radiation, ionizing radiation, and hydroxyurea.[30−32] Cisplatin treatment also led to lysis of *Escherichia coli* cells containing bacteriophage *λ*,[33] another result shared by DNA−damaging agents. These early experiments pointed to DNA as an important cellular target.

An examination of the inhibitory effects of platinum compounds on DNA, RNA, and protein synthesis provided additional information about the cellular target of cisplatin. The incorporation of radiolabeled precursors into RNA, DNA, and proteins was studied in both human amnion $AV_3$ cells in vitro and Ehrlich ascites tumor cells in vivo.[34,35] DNA synthesis was selectively inhibited compared to protein and RNA synthesis. The amount of platinum bound to these macromolecules was examined in HeLa cells in conjunction with a colony-forming assay.[36] The number of platinum atoms bound when the surviving fraction of cells was reduced by a given amount was determined for DNA, RNA, and proteins. These results indicated that 22 platinum atoms were bound per DNA molecule compared to one Pt per mRNA, one per 30 rRNA, one per 1500 tRNA, and one per 1500 protein molecules. In a more recent study, the number of platinum atoms binding to DNA, RNA, and protein molecules was measured in HeLa cells treated with $^{195m}$Pt-radiolabeled cisplatin at its mean lethal concentration.[37] Cells were then fractionated, and the number of platinum atoms bound to each macromolecule was calculated. The results showed that 1 out of $3 \times 10^4$ to $3 \times 10^5$ protein molecules contained a platinum atom, whereas between one in 10 and one in 1000 RNA molecules contained a platinum atom. In contrast, the DNA had nine platinum atoms bound per molecule, further indicating it to be the important cellular target. Other studies have examined the effects of cisplatin on repair-deficient *E. coli* and human cells.[38−44] In these experiments, the repair-deficient mutants were more sensitive to cisplatin treatment compared to the wild-type cells. These results suggested that cisplatin damages DNA in these cells and that their differential ability to repair this damage leads to differences in cisplatin sensitivity.

This experimental evidence provides a strong case that DNA is the primary target of cisplatin in cells and has directed the mainstream of research accordingly. There is some evidence to suggest that non-DNA targets may be involved, and this topic has recently been reviewed.[45] Cisplatin interacts with phospholipids and phosphatidylserine in membranes,[46,47] disrupts the cytoskeleton,[48] and affects the polymerization of actin, presumably because of conformational changes resulting from the formation of Pt−S bonds.[49] The preference for Pt−N bond formation in the presence of S-donor ligands in cells is discussed elsewhere in this issue. Thus, although there is some evidence to suggest that other biological targets may be important in the cisplatin mechanism, it is generally accepted that DNA is the primary target, and research in this area has predominated.

## B. DNA Adducts Formed by Cisplatin

Once DNA was established as the primary target of cisplatin in cells, details of the binding interaction were investigated to characterize the types of adducts formed. Figure 2 illustrates structurally characterized binding modes. Bifunctional adducts involving either intrastrand or interstrand cross-links on DNA or protein−DNA cross-links were thought to be

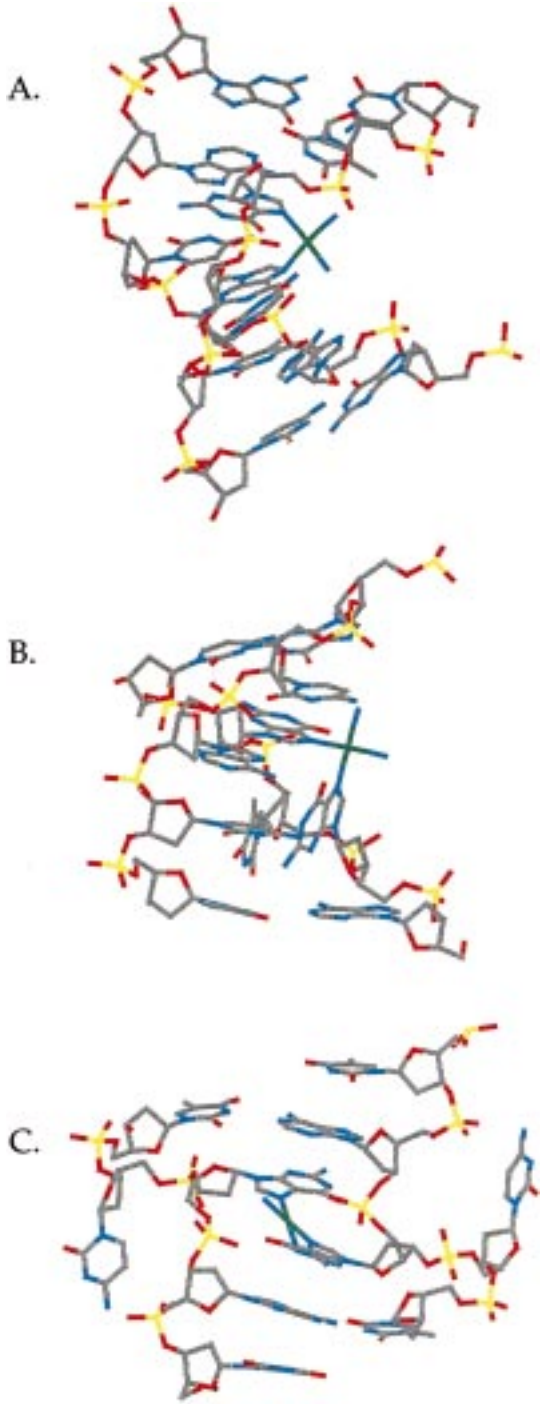


**Figure 2.** Diagram of cisplatin−DNA adducts. A depicts a 1,2-intrastrand cross-link,[87,88] B a 1,3-intrastrand cross-link,[95] and C an interstrand cross-link.[96] Monofunctional adducts and protein−DNA cross-links can also be formed.

important for biological activity because [Pt(dien)Cl]$^+$ and related inactive compounds only bind in a monofunctional manner.[50] Enzymatic digestion of cisplatin-treated salmon sperm DNA followed by chromatographic separation of the products and $^1$H NMR analysis allowed the identification of the major DNA adducts formed by cisplatin.[51] The major products were 1,2-intrastrand cross-links involving adjacent bases, with *cis*-[Pt(NH$_3$)$_2$\{d(GpG)\}] (*cis*-GG) comprising 47−50% of the adducts formed and *cis*-[Pt(NH$_3$)$_2$\{d(ApG)\}] (*cis*-AG) comprising another 23−28%. In addition, 8−10% of the digested products contained 1,3-intrastrand cross-links involving nonadjacent guanines (*cis*-GNG) and interstrand adducts, and another 2−3% of the products was the result of monofunctional binding to guanine.[51] In all cases, platinum was bound to the N7 atom of purine bases. In a similar study of DNA modified by [$^3$H]-[Pt(en)Cl$_2$], a compound expected to have a similar adduct profile to cisplatin, there were 65% *cis*-GG adducts, 25% *cis*-AG, and 6% *cis*-GNG adducts.[52]

The results of these in vitro experiments provided evidence that the 1,2-intrastrand cross-links were the major adducts formed by cisplatin. By using immunochemical methods, the DNA adduct profile in cisplatin-treated cells was examined. A study using white blood cells taken from cancer patients treated with cisplatin displayed a similar adduct profile to the in vitro studies described above.[53] For one patient, there were 65% *cis*-GG adducts and 22% *cis*-AG adducts present. In another study, the level of intrastrand adducts measured in cells of patients treated for ovarian and testicular cancer was correlated to treatment response.[54−56]

One of the interesting differences between cisplatin and its clinically ineffective isomer, *trans*-DDP, is their ability to form different types of DNA cross-links. Both compounds form bifunctional DNA adducts, binding to the N7 positions of guanine and adenine. *trans*-DDP is unable to form 1,2-intrastrand d(GpG) or d(ApG) adducts, however, due to its stereochemistry.[57] Enzymatic digestion studies of DNA treated with *trans*-DDP, similar to the ones with cisplatin described above, indicated the main products to be 50% *trans*-[Pt(NH$_3$)$_2$\{d(GMP)\}-\{d(CMP)\}], 40% *trans*-[Pt(NH$_3$)$_2$\{d(GMP)$_2$], and 10% *trans*-[Pt(NH$_3$)$_2$\{d(GMP)\}\{d(AMP)\}].[58] In the product containing cytosine, the platinum coordinated to the N3 position of the base. These results reflect formation of 1,3-intrastrand and interstrand cross-links.

Experiments have demonstrated that cisplatin binding to DNA is kinetically rather than thermodynamically controlled. As mentioned above, a chloride ligand hydrolyzes when cisplatin enters cells, forming *cis*-[Pt(NH$_3$)$_2$Cl(H$_2$O)]$^+$. This hydrolysis reaction is the rate-limiting step for DNA binding, the half-life being ∼2 h.[59−61] Aquated cisplatin subsequently binds to an N7 atom of a guanine base in DNA, which displaces the water molecule in a relatively fast reaction step ($t_{1/2}$ ∼ 0.1 h), forming a monofunctional adduct.[59,61] Closure of the monofunctional adduct to form a bifunctional adduct involves hydrolysis of the second chloride ligand, with a half-life of ∼2 h.[59,61] Interestingly, the first hydrolysis step and subsequent binding of both cisplatin and *trans*-DDP to DNA to form monofunctional adducts occur at a similar rate. The rate of closure to form a bifunctional adduct, however, is controversial. Some studies revealed rates to be similar for the two compounds ($t_{1/2}$ ∼ 3 h),[59] whereas others determined that *trans*-DDP forms bifunctional adducts much more slowly.[62,63] Differences in the rate of closure could explain the different biological activities of the two compounds. The discrepancy in closure rates to form *trans*-DDP bifunctional adducts may reflect differences in the samples used; the length of the DNA duplex and the presence of nearby platinum adducts can affect the rate of closure.[63] At low platination levels, closure to bifunctional adducts appears to occur slowly ($t_{1/2}$ > 24 h), implying that this difference, which may derive from the inability of *trans*-DDP to form 1,2-intrastrand cross-links, may contribute to the different clinical responses of these two compounds.[63]

The results of the experiments just described suggest that the 1,2-intrastrand adducts of cisplatin may be important to its anticancer activity. Not only are these major adducts formed both in vitro and in vivo, but the failure of clinically inactive compounds to form such cross-links is also a notable difference. Another indication that these adducts are important comes from the positive correlation of the level of these cisplatin adducts to treatment response. Therefore, many studies have focused on examining their structure and biological activity in order to determine the role they may play in the cisplatin anticancer mechanism.

## C. Structural Studies of Cisplatin−DNA Adducts

The formation of cisplatin−DNA cross-links structurally distorts the DNA. Initial work showed that cisplatin binding could unwind DNA and, at saturation levels, shorten the duplex by up to 50%.[64−66] The formation of these adducts also results in a loss of helix stability, as demonstrated by calorimetric studies on calf thymus and linearized plasmid DNA[67−69] and gradient denaturing gel electrophoresis studies of the *cis*-GG adduct.[70] Further calorimetric experiments with site-specific cisplatin−DNA adducts revealed a duplex destabilization of 6.3 kcal/mol associated with *cis*-GG adduct formation.[71] The identity of the bases flanking the platination site has very recently been shown to modulate the extent of this destabilization.[72] Much work has been performed to learn about the structure of the various cisplatin−DNA adducts and has been recently reviewed.[73,74]

Early structural studies used NMR and X-ray crystallography to examine single-stranded DNA fragments containing a cisplatin adduct.[50] X-ray crystallography revealed the nature of the *cis*-GG cross-link on the dinucleotide d(pGpG)[75,76] and trinucleotide d(CpGpG).[77] The d(pGpG) structure, shown in Figure 3, has the two guanine rings oriented in a "head-to-head" configuration, with the two O$_6$ atoms on the same side of the platinum coordination plane. The dihedral angle between the guanine rings ranges from 76° to 87°, reflecting destacking of the bases. Another notable feature of this structure is that one

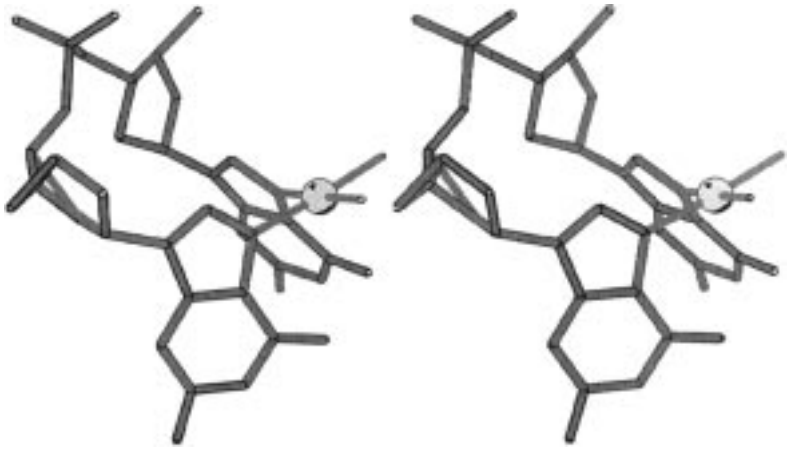

**Figure 3.** Stereoview of a MOLSCRIPT[329] representation of the X-ray crystal structure of *cis*-[Pt($NH_3$)$_2${d(pGpG)}].[75,76]

of the platinum ammine ligands is hydrogen bonded to an oxygen atom on the 5′-phosphate group. This interaction may be an important feature, potentially stabilizing these DNA adducts. NMR work combined with molecular mechanics calculations on duplex DNA containing a cisplatin 1,2-intrastrand d(GpG) adduct showed that the adduct caused the helix to bend ∼60° toward the major groove.[78−80] More recently, a novel "head-to-head" conformer of the d(GpG) cross-link was identified in studies with [Pt(2,2′-bipiperidine)$Cl_2$] by using NMR, HPLC, and mass spectrometric methods.[81] The major difference between the two d(GpG) conformers is the propagation direction of the phosphodiester linkage (Figure 4). Molecular mechanics and dynamics calculations indicate that, although it is possible for this new conformer to form with cisplatin, it is unlikely to exist in a duplex at low temperature. It is hypothesized that the conformer could potentially be important in mutational events, duplex breathing, or duplex interactions with cellular proteins.

Gel electrophoresis studies were also employed to gain structural information about the various site-specific cisplatin−DNA adducts. The first studies used multimers of a 22-bp oligonucleotide containing a 1,2-intrastrand d(GpG) cisplatin cross-link.[82] These experiments showed the DNA to bend ∼40° in the direction of the major groove and were extended to examine the DNA bending and unwinding induced by other adducts of cisplatin and *trans*-DDP.[83,84] The *cis*-GG and *cis*-AG cross-links bent the helix by 32°−34° and unwound it by 13°, whereas the *cis*-GNG adduct bent DNA by ∼35° and unwound it by 23°. Although, the 1,3-intrastrand d(GpNpG) adduct of *trans*-DDP bent the DNA, a flexibility was imparted to the DNA which acted like a hinge joint without producing a directed bend. In similar studies performed with interstrand cross-links formed by cisplatin binding to two guanines, the DNA was bent by ∼45−55° toward the major groove and unwound by ∼79°.[85,86]

This work was followed by several high-resolution X-ray and NMR structural studies of the cisplatin adducts (see Tables 1 and 2). Figure 5 presents the 2.6 Å resolution X-ray crystal structure of a DNA dodecamer containing a 1,2-intrastrand d(GpG) cisplatin adduct.[87,88] The helix bends by ∼50° toward

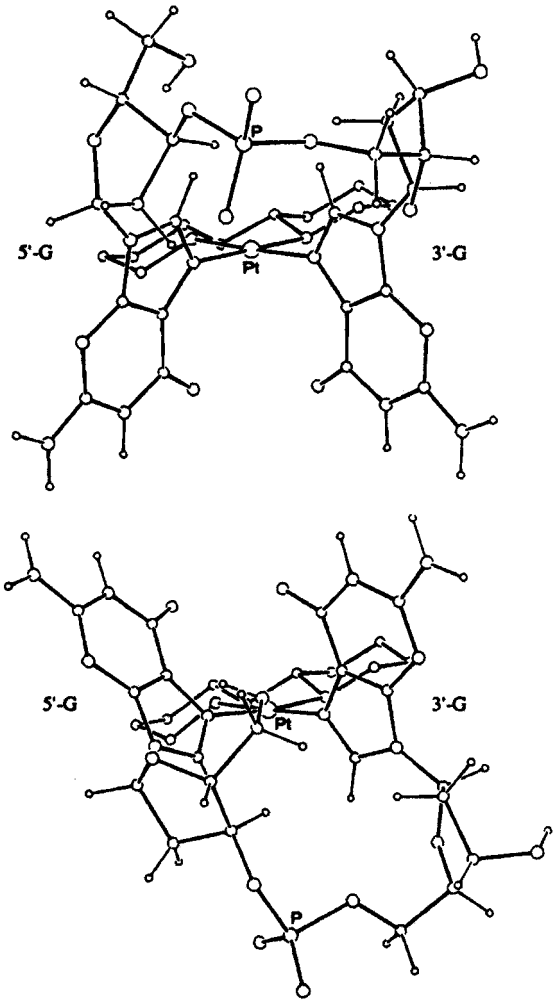


**Figure 4.** View of the two head-to-head conformers of *cis*-[Pt(2,2′-bipiperidine){d(GpG)}]. Reprinted with permission from ref 81. Copyright 1998 American Chemical Society.

the major groove, and the dihedral angle between the guanine bases is 30°, considerably less than in the d(pGpG) structure mentioned above. One of the ammine ligands bound to platinum is hydrogen bonded to a phosphate oxygen, as seen previously. The base pairs at the platination site are propeller twisted, but retain their hydrogen bonds. The platinum atom is displaced from the planes of the guanine rings by ∼1 Å, resulting in a strained environment.

**Table 1. Selected Structural Features of Cisplatin−DNA Adducts**

| DNA sequence | method | bend angle (deg) | average helical twist[a] (deg) | Pt site | unwinding[a] (deg) | ref |
|---|---|---|---|---|---|---|
| d(CCTCTG*G*TCTCC)· d(GGAGAC C AGAGG) | X-ray | 39–55 | 32 | major groove | nd | 87,88 |
| d(CCTCTG*G*TCTCC)· d(GGAGAC C AGAGG) | NMR | 78 | 27 | major groove | nd | 89 |
| d(CCTG*G*TCC)· d(GGAC C AGG) | NMR | 58 | 25[b] | major groove | 21 | 90 |
| d(GACCATATG*G*TC)· d(GACCATATG*G*TC) | NMR | 40 | nd | major groove | nd | 91 |
| d(CTCTCG*G*TCTC)· d(GAGACC G AGAG) | paramagnetic NMR | ~80 | 26[b] | major groove | nd | 93 |
| d(CTCA*G*CCTC)· d(GAGG CT GAG) | NMR and molecular modeling | 55 | nd | major groove | nd | 94 |
| d(CTCTAG*TG*CTCAC)· d(GTGAGC AC TAGAG) | NMR | 20–24 | nd | major groove | 19 | 95 |
| d(CCTCG*CTCTC)· d(GAGAG*CGAGG) | X-ray | 47 | nd | minor groove | 70 | 98 |
| d(CATAG*CTATG)· d(CATAG*CTATG) | NMR | 20 | nd | minor groove | ~80 | 96 |
| d(CCTCG*CTCTC)· d(GAGAG*CGAGG) | NMR | 40 | nd | minor groove | 76 | 97 |

[a] Values not determined are denoted as nd. [b] Value taken from ref 74.

**Table 2. Structural Parameters for X-ray and NMR Solution Structures of DNA Duplexes Containing the 1,2-Intrastrand d(GpG) Cisplatin Adduct (adapted from ref 74)**

| | X-ray | | NMR | | |
|---|---|---|---|---|---|
| parameter | refs 87,88 | ref 295[a] | ref 89 | ref 90 | ref 93 |
| DNA length (bp) | 12 | 16 | 12 | 8 | 11 |
| DNA form | A/B junction | B | B | B | B |
| minor groove width (Å) | 9.5−11.0 | 5.5−12.0 | 9.4−12.5 | 4.5−7.8 | 9.0−12 |
| minor groove depth (Å) | 3.0 | na | 1.4 | 3.2 | 2.1 |
| average P−P distance (Å) | 5.5 | na | 6.8 | 6.8 | 6.8 |
| dihedral angle between platinated bases (deg) | 30 | 75 | 47 | 59 | 58 |
| average helical twist (deg) | 32 | 33 | 27 | 25 | 26 |
| DNA bend[b] (deg) | 39 and 55 | 61 | 78 | 58 | 81 |

[a] Values not available are denoted as na. [b] Values determined with the program Curves.[328]

The overall conformation of the DNA is a fusion of the A-form and B-form structure types, possibly as a consequence of crystal packing forces. The DNA has a wide and shallow minor groove, an important recognition element for protein binding (vide infra).

The structure of DNA containing 1,2-intrastrand d(GpG) cisplatin adducts has also been determined by NMR methods (see Table 2). In an interesting comparison to the X-ray study just described, the NMR solution structure of the same platinated DNA dodecamer was determined (Figure 6).[89] Here the overall helix bend was 78°, and the dihedral angle between adjacent guanine bases was 47°. These values are larger than observed for the X-ray structure, reflecting the influence of crystal packing in the latter study. The base pairing at the site of platination is also more distorted in the NMR solution structure. The platinum is displaced by 0.8 Å from the planes of the guanine rings. The DNA has a flat, wide minor groove, and in general, the global curvature of the helix is very similar to that of the duplex in the X-ray crystal structure. The NMR solution structure of an octamer duplex with a 1,2-intrastrand d(GpG) cisplatin adduct has also been reported.[90] This helix has the DNA bent by 58° toward the major groove, and the dihedral angle between the guanine base planes is 59°. The platinum is displaced from the planes of the guanine bases, and the minor groove is widened, although not to the same extent as in the dodecamer structures. Finally, the NMR solution

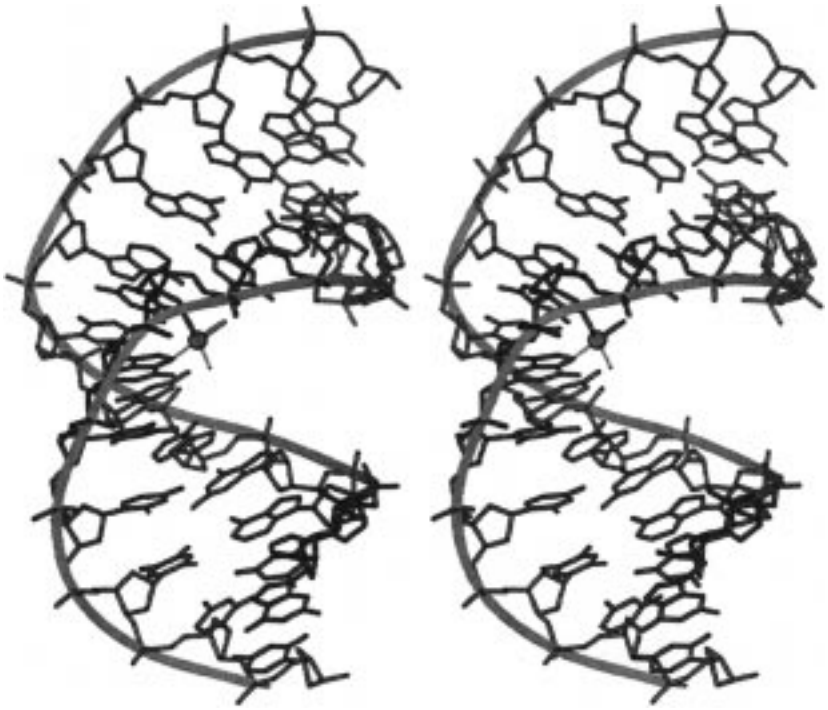

**Figure 5.** Stereoview of a MOLSCRIPT[329] representation of the X-ray crystal structure of d(CCTCTG*G*TCTCC)·d(GGAGACCAGAGG) containing a *cis*-GG adduct, where G* denotes the location of platinated nucleotides.[87,88]

**Figure 6.** Stereoview of a MOLSCRIPT[329] representation of the NMR solution structure of d(CCTCTG*G*TCTCC)·d(GGAGACCAGAGG) containing a *cis*-GG adduct, where G* denotes the location of platinated nucleotides.[89]

structure of a palindromic dodecamer DNA probe with two 1,2-intrastrand d(GpG) adducts positioned to be 180° apart from each other in B-form DNA was determined.[91] Each *cis*-GG adduct bends the DNA by ~40°, and the helix axis is dislocated by ~13 Å.

In the traditional NMR studies described above, distance constraints from nuclear Overhauser effect (NOE) data are used in the structure determination. The NOE data provide short-range distance (≤5 Å) information, which is useful for addressing local geometry but not for providing any information about long-range interactions which may be important in determining the structure of a biomolecule. To address this issue, a paramagnetic moiety can be incorporated into a macromolecule to afford long-range (10−30 Å) electron−proton distance constraints. Toward this end, a cisplatin analogue containing a 4-amino-TEMPO (4-amino-2,2,6,6-tetramethylpiperidinyloxy, free radical) ligand was prepared to study the structure of the *cis*-GG adduct. In preliminary work, the binding of this compound to d(GpG) was investigated, and the NMR spectrum

**Figure 7.** Stereoview of a MOLSCRIPT[329] representation of the NMR solution structure solved with paramagnetic constraints of d(CTCTCG*G*TCTC)·d(GAGACCGAGAG) containing a *cis*-[Pt($NH_3$)(4-aminoTEMPO){d(GpG)}] cross-link, where G* denotes the location of platinated nucleotides.[93]

**Figure 8.** Stereoview of a MOLSCRIPT[329] representation of the NMR solution structure of d(CTCTAG*TG*CTCAC)·d(GTGAGCACTAGAG) containing a *cis*-GTG adduct, where G* denotes the location of platinated nucleotides.[95]

was analyzed to produce a structure comparable to the X-ray structure of cisplatin bound to d(pGpG).[92] The strategy was then applied to investigate an undecamer DNA duplex containing a *cis*-GG adduct (Figure 7).[93] In the resulting structure, the helix bend angle is ~80°, and there is a wide minor groove, similar to that in the X-ray and NMR structures of the dodecamer. The use of the long-range distance constraints resulted in more information about the conformation of the DNA, especially at the 5′-end of the duplex. The structure determined by this methodology is similar to that of the dodecamer, as determined by X-ray crystallography, and those of the dodecamer and octamer, as revealed by NMR spectroscopy.

Although most of the structural work has been performed on DNA containing the major *cis*-GG adduct, there have also been studies of other cisplatin

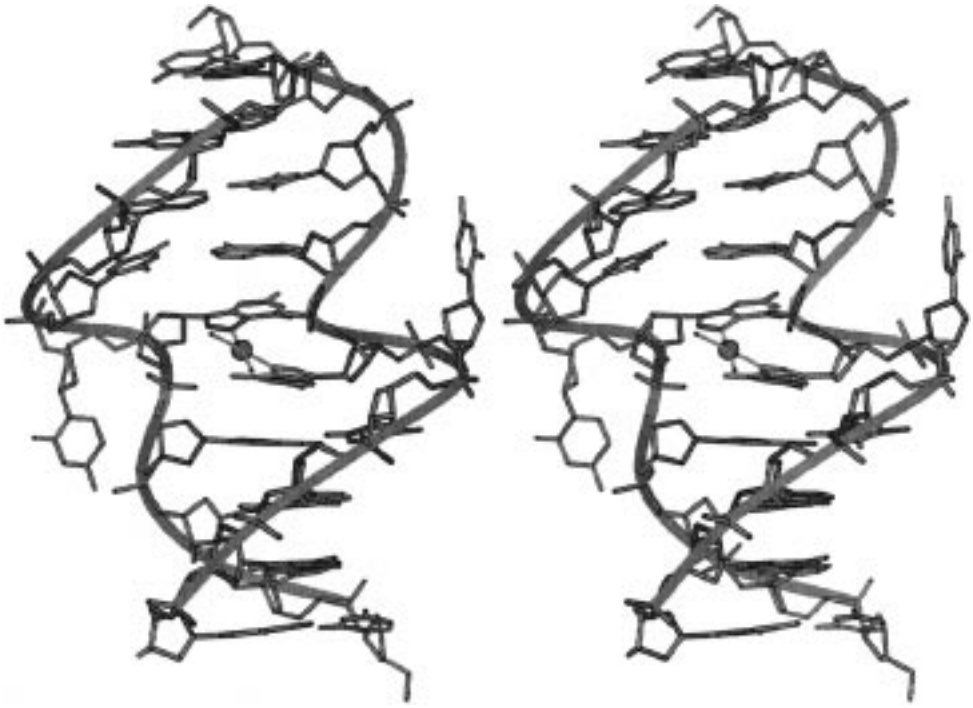

**Figure 9.** Stereoview of a MOLSCRIPT[329] representation of the NMR solution structure of d(CATAG*CTATG)·d(CATAG*CTATG) containing a cisplatin interstrand cross-link, where G* denotes the location of platinated nucleotides.[96]

adducts (see Table 1). Whereas no high-resolution structure is available, there is some geometric information for a nonanucleotide containing a 1,2-intrastrand d(ApG) cisplatin adduct. NMR data suggest that this oligonucleotide is kinked at the platination site in a manner similar to that observed for a *cis*-GG adduct, and the minimized structure in molecular modeling studies indicates that this duplex bends by ~55° toward the major groove.[94] The 1,3-intrastrand d(GpNpG) cisplatin adduct has also been studied by NMR methods (Figure 8).[95] The structure of a 13-bp probe containing a 1,3-intrastrand d(GpTpG) adduct shows a very small global helix curvature of ~20°–24°. The helix is unwound by ~19°. Unlike the structures of the 1,2-intrastrand adducts, the base pairing around the cisplatin adduct is severely disrupted. The significant structural differences between this adduct and the major cisplatin 1,2-intrastrand adducts suggest that their biological processing will be different.

The interstrand DNA cross-link formed by cisplatin has also been a subject of study by NMR and X-ray methods. To date, the NMR structures of two different DNA decamers containing interstrand cross-links, where the platinum is bound to two guanine bases, have been determined.[96,97] One of these structures is illustrated in Figure 9.[96] A surprising feature of these structures is that the *cis*-diammineplatinum(II) moiety is located in the minor groove. The interstrand adduct unwinds the DNA by ~80° in both structures, and the helix bends by ~20°–40° toward the minor groove. The X-ray crystal structure of one of these decamers has been determined at 1.63 Å resolution.[98] The unusual placement of the *cis*-diammineplatinum(II) moiety in the minor groove of the DNA observed in the NMR determination is also observed in this crystal structure. The DNA helix is bent by 47° toward the minor groove and is unwound by 70°. Interestingly, the X-ray crystal structure of a DNA base excision repair product of the G:T/U-specific mismatch DNA glycosylase (MUG) has a very similar structure to that of the duplex containing the cisplatin interstrand cross-link.[99] Base excision is used to repair different types of DNA damage, including uracil bases, alkylated purines, and G:T mismatches. The N-glycosidic bond is hydrolyzed by a DNA glycosylase, releasing the damaged base, a process followed by excision of the abasic sugar ring. The similarity of these structures suggests that cisplatin interstrand cross-links might be recognition elements for proteins that recognize extrahelical nucleotides or abasic sites in DNA, such as mismatch repair proteins[100] or AP endonucleases.[101,102] This hypothesis has not yet been evaluated experimentally.

The high-resolution structures of the various cisplatin–DNA adducts just described definitively reveal their propensity to distort DNA in very different ways. The major 1,2-intrastrand cross-links afford a bent, unwound duplex with a widened, shallow minor groove unlike that displayed by the minor cisplatin adducts. Their disparate structural features suggest different roles for these various adducts in mediating the antitumor properties of cisplatin.

## D. Alternative Cellular DNA Targets

Most cisplatin research has focused on its ability to modify genomic DNA (gDNA) in the nucleus. Recently an alternative cellular DNA target has been identified which could potentially play a role in the cisplatin mechanism. Mitochondrial DNA (mtDNA), which lacks histones,[103] has been targeted by DNA-damaging agents such as methylnitrosourea, aflatoxin B1, and bleomycin.[103–105] A study in Chinese hamster ovary cells used a dissociation-enhanced

lanthanide fluoroimmunoassay, DELFIA, and immunoelectron microscopy to determine the levels of cisplatin−DNA adducts.[106] There was a 6- and 4-fold higher proportion of adducts in mtDNA, compared to gDNA, as revealed by the DELFIA and microscopic methods, respectively. Similar results occurred in rat and monkey tissues after transplacental cisplatin exposure.[107,108] The preference for mtDNA was later attributed both to higher initial binding and to the lack of removal of the cisplatin−DNA adducts.[109] The persistence of cisplatin adducts on mtDNA may be due to the inability of mitochondria to perform nucleotide excision repair, a major pathway for removing cisplatin damage in gDNA.[104] Thus, cisplatin binding to mtDNA may contribute to its anticancer mechanism.

## III. Effect and Consequences of Platinum on DNA Function

The ability of cisplatin to bind to DNA and distort its structure suggested that it would interfere with the normal functioning of this important cellular component. DNA replication and transcription are essential for cell division and protein production; any disruption in these processes would be cytotoxic. For these reasons, the effects of platination on DNA function have been extensively investigated with the aim of better understanding the biological activity of this drug.

### A. Cisplatin Effects on DNA Replication

The inhibition of DNA synthesis by cisplatin was discovered early and suggested DNA to be the principal cellular target of the drug. Replication is an essential cellular process that involves unraveling of double-stranded DNA from chromatin, separation of the duplex strands, and the synthesis of new DNA using the original strands as templates. DNA polymerases are integrally involved in this process. The inhibition of replication by cisplatin suggested that it might kill cancer cells by blocking their ability to synthesize new DNA required for division. With the use of salmon sperm DNA or poly[d(A−T)·d(A−T)] as a template, the activity of partially purified human DNA polymerases α and $\beta$ and Rauscher murine leukemia virus reverse transcriptase was inhibited when the DNA was modified with cisplatin or *trans*-DDP.[110] The amount of platinum bound per nucleotide required to inhibit 50% of the activity was 2−7-fold less for cisplatin compared to *trans*-DDP, depending on the DNA source. A similar study used an in vitro T7 DNA replication system to reveal that cisplatin inhibited DNA replication better than *trans*-DDP by ∼5-fold.[111] The DNA template in this study was incubated with cis- and *trans*-DDP for 3 h at 37 °C, which may not be a long enough for *trans*-DDP to form bifunctional adducts.[62,63] The ability of cisplatin to block DNA replication in these early experiments suggested that this activity may be important to the mechanism of cytotoxicity.

Further studies used the large (Klenow) fragment of DNA polymerase I and a single-stranded M13 mp8 DNA template to examine second-strand synthesis as a model for DNA replication.[112] Both cisplatin and *trans*-DDP blocked DNA synthesis in a sequence-specific manner, with cisplatin preferring all $(dG)_n$ ($n \geq 2$) sites. *trans*-DDP was less selective, with d(GpNpG) sequences being the most prevalent. The $[Pt(dien)Cl]^+$ cation, which can only form monofunctional DNA adducts, did not block DNA synthesis. The results of this study were extended in later work to investigate the GC box element of simian virus 40 (SV40) DNA, which contains tandem repeats of the sequence GGGCGG that proved to be a good target for cisplatin.[113] The GC box is important for viral DNA replication and for expression of the viral transforming gene. The ability of cisplatin to stop DNA polymerase I at the GC box implies that it may be able to target similar sequences in retroviruses and oncogenes, contributing to the activity of the drug. In fact, cisplatin-resistant mutants of SV40 were isolated that had acquired specific deletions within the GC box region.[114] This sequence-specificity of DNA synthesis inhibition was similar when using enzymes from prokaryotes and eukaryotes; *E. coli* DNA polymerase I blocked synthesis on single-stranded, cisplatin-treated DNA at similar sites to DNA polymerase α from *Drosophila melanogaster* embryos or calf thymus.[115,116] In these studies *cis*-GG adducts, formed by global platination of the DNA, inhibited replication better than *cis*-AG adducts.[116] DNA polymerase $\epsilon$, isolated from calf thymus, was also blocked by cisplatin, and its adducts were suggested to interfere with the DNA repair functions of this enzyme.[117]

The effect of cisplatin on DNA replication was examined by using the SV40 chromosome in green monkey CV-1 cells.[118] Fourteen times more *trans*-DDP than cisplatin was required to inhibit DNA replication to the same extent in this system. At doses producing equal amounts of DNA replication inhibition, however, the amount of platinum bound per SV40 DNA was identical for *trans*-DDP and cisplatin. Similar results were obtained for SV40 replication using extracts from human HeLa and 293 cells.[119] One explanation for the higher dose of *trans*-DDP required to produce the same amount of inhibition is that DNA adducts formed by the two compounds are differentially repaired.[118] Experiments in HeLa and 293 cells revealed enhanced repair activity selective for *trans*-DDP-modified DNA consistent with this idea,[119] but other studies with Chinese hamster and African green monkey cells were unable to find evidence for the selective repair of *trans*-DDP adducts.[120] Another difference between cisplatin and *trans*-DDP was detected in a study examining various functions of *E. coli* DNA polymerase I,[121] an enzyme having DNA synthesis, 3′−5′-exonuclease proofreading, and 5′−3′-exonuclease repair activities. Both the initial rate and total excision were inhibited for the 5′−3′-exonuclease with cisplatin treatment, whereas with *trans*-DDP the activity was basically unaffected. It was suggested that this difference could explain the higher concentrations of *trans*-DDP needed to inhibit bacterial growth compared to cisplatin.

M13 genomes site-specifically modified with cisplatin were used to assess the ability of different

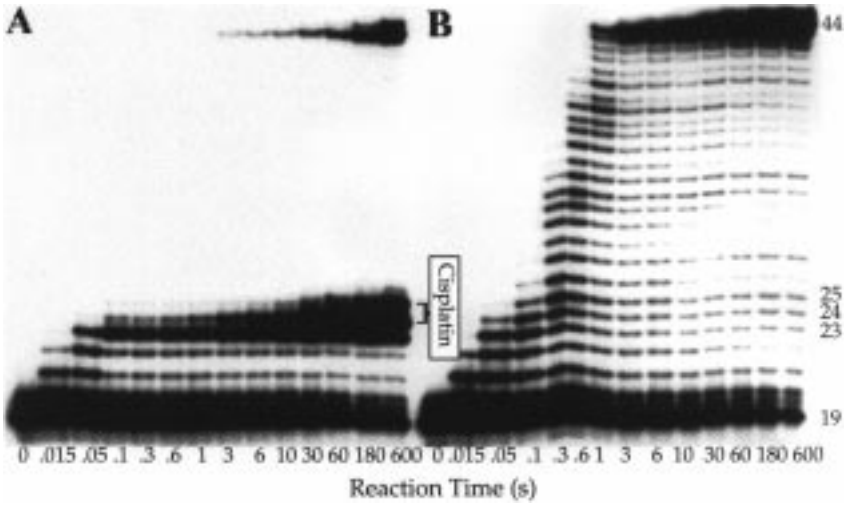


**Figure 10.** Gel electrophoresis data obtained during a kinetic study of the effect of a *cis*-GG adduct on DNA polymerization by HIV-1 reverse transcriptase. Panel A shows fragments generated by enzymatic replication of a 44-bp $^{32}$P-end-labeled DNA duplex containing a site-specific *cis*-$\{Pt(NH_3)_2\}^{2+}$ cross-link at G(24)/G(25). Polymerization is blocked by platination of the substrate. Panel B depicts results for an unmodified DNA probe. Similar results were seen with T7 DNA polymerase. Reprinted with permission from ref 123. Copyright 1999 American Chemical Society.

cisplatin−DNA adducts to block replication in vitro.[122] This study examined the *cis*-GG, *cis*-AG, and *cis*-GNG adducts and employed a number of different DNA polymerases, including DNA polymerase I, bacteriophage T7 DNA polymerase, bacteriophage T4 DNA polymerase, Taq polymerase, and DNA polymerase III. The results indicated that, on average, the polymerases were able to bypass the cisplatin adducts ~10% of the time. The *cis*-GG adduct was the most inhibitory lesion. The frequency of replication bypass varied for the different polymerases, with bacteriophage T4 DNA polymerase being the most strongly inhibited. For this enzyme, all of the cisplatin adducts were bypassed only ~2% of the time. The results of this study showed that polymerases can bypass cisplatin adducts and suggested that the drug may induce mutagenesis through such replication bypass.

Kinetic studies of the effect of a *cis*-GG adduct on DNA polymerization were recently undertaken by using single turnover kinetic methods.[123] Both T7 DNA polymerase and HIV-1 reverse transcriptase activities exhibited strong pauses during DNA synthesis at sites corresponding to one nucleotide preceding the first platinated guanine residue and at the positions opposite the two platinated guanines (Figure 10). DNA polymerization occurred with biphasic kinetics. A small amount of DNA was productively bound and able to undergo fast polymerization. The majority of the population bound in a nonproductive manner, leading to slow polymerization. The distortion of DNA base pairs at the site of platination was suggested to alter the alignment of DNA in the binding site of the T7 DNA polymerase, slowing the protein conformational change necessary for polymerization. The binding of the next correct nucleotide was also affected by the presence of the cisplatin adduct.

The results of the studies discussed above demonstrate that cisplatin can affect DNA replication. When similar amounts of platinum are bound in a bifunctional manner to the DNA, however, there does not appear to be large difference between cisplatin and the clinically inactive *trans*-DDP. Rather, differences in the concentrations of the compounds required to inhibit DNA replication appear to be the result of differential cellular processing. Thus, whereas inhibition of DNA replication may be part of the cisplatin mechanism, it cannot fully explain the anticancer properties of the drug.

## B. Cisplatin Effects on DNA Transcription

Studies designed to investigate the relationship between the inhibition of DNA synthesis, cytotoxicity, and cell cycle progression suggested that cisplatin might inhibit DNA transcription.[124,125] Transcription is the cellular process whereby mRNA is produced from a DNA template, a critical step in protein synthesis. In these studies, cells treated with cisplatin progressed through the S phase, where DNA synthesis occurs, and were arrested in the $G_2$ phase (Figure 11).[124,125] This $G_2$ arrest was temporary for cells treated with low concentrations of cisplatin, but cells treated with higher doses remained in $G_2$ arrest

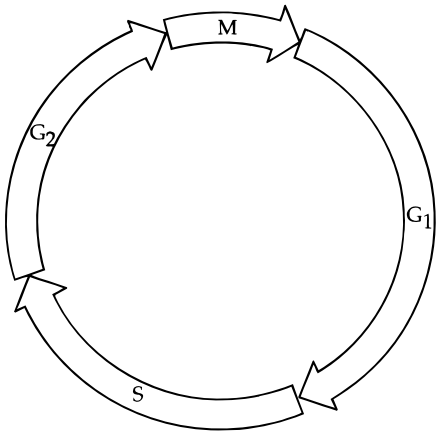


**Figure 11.** A representation of the cell cycle. $G_1$ is the first gap phase, S is the DNA synthesis phase, $G_2$ is the second gap phase, and M is mitosis. This figure is based on one in ref 330.

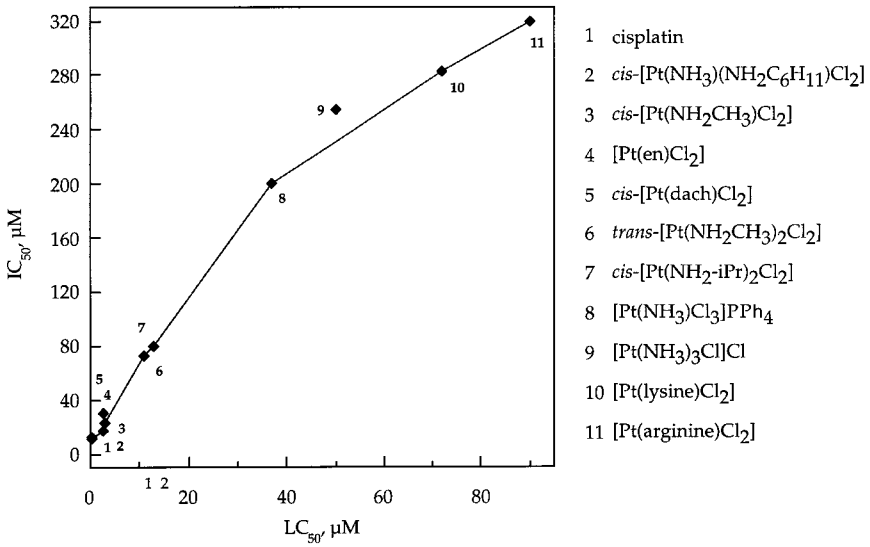


**Figure 12.** Correlation of $IC_{50}$ with $LC_{50}$ from the EGFP reporter gene assay. $LC_{50}$ is the concentration of compound that kills 50% of the cells, and $IC_{50}$ is the concentration of compound that reduces EGFP expression by 50% of the control value. Reprinted with permission from ref 139. Copyright 1999 Elsevier Science Ltd.

until cell death occurred. Work using DNA excision repair proficient and deficient Chinese hamster ovary cells was performed in order to study the relationship between DNA replication and cellular toxicity.[125] In these studies, inhibition of DNA synthesis depended only on cisplatin concentration and was not related to the sensitivity of the cell line to cisplatin, demonstrating that replication inhibition did not correlate directly with cisplatin toxicity. The ability of cisplatin to arrest the cell cycle in $G_2$, however, was related to the sensitivity of the cell line. The repair deficient cells were arrested in $G_2$ at lower cisplatin concentrations than the repair proficient cells. These results are consistent with the hypothesis that $G_2$ arrest results from the inability of the cells to transcribe genes necessary to enter mitosis. The ability to perform DNA repair removes cisplatin adducts that block transcription, allowing cell cycle progression. The results of these experiments indicated that cisplatin cytotoxicity might be due to more than simple inhibition of DNA synthesis and implicated DNA transcription in the anticancer mechanism.

The ability of specific cisplatin adducts to inhibit transcription was studied more directly in later work using wheat germ RNA polymerase II and *E. coli* RNA polymerase.[126−128] Transcription elongation was blocked for these polymerases by the *cis*-GG, *cis*-AG, *cis*-GNG, and cisplatin interstrand cross-links. Neither *trans*-DDP nor monofunctional adducts could provide an absolute block for these enzymes. In other experiments, transcription by T7 and SP6 RNA polymerases from a template DNA restriction fragment modified with cisplatin was examined.[129] These enzymes were also blocked at *cis*-GG and *cis*-AG sites. The ability of cisplatin adducts to inhibit RNA polymerase II initiation and elongation in human cell extracts has recently been reported.[130] Initiation of transcription was inhibited by increasing concentrations of cisplatin. The presence of cisplatin−DNA damage on an exogenous plasmid inhibited transcription initiation from an undamaged template. This result is consistent with the hypothesis that proteins necessary for transcription to occur may be hijacked away from their normal binding sites by cisplatin-damaged DNA. The ability of RNA polymerase II to bypass site-specific cisplatin−DNA adducts during transcription elongation was also examined. Here, the polymerase was able to bypass the *cis*-GG adducts, whereas it was blocked by *cis*-GNG adducts. This unexpected result was not obtained with T3 RNA polymerase, which was blocked effectively by both types of cisplatin cross-link. Further investigation of the relative abilities of cisplatin−DNA adducts to block transcription by mammalian RNA polymerases seems warranted.

The regulation of gene expression by cisplatin has also been studied. Test expression genes were introduced by transient transfection into CV-1 monkey cells.[131] When the cells were treated with cisplatin, there was a strong differential inhibition of the genes. Different promoters were studied, and stronger inhibition was observed for the stronger promoters. Both strong and weak promoters were equally insensitive to transcription inhibition by *trans*-DDP. In similar experiments with transient and stably transfected HeLa cells, expression from some of the weaker promoters was strongly induced, whereas the stronger promoters were strongly inhibited.[132] These results suggest that the strong promoters are associated with accessible chromatin and therefore more easily modified by cisplatin. The expression of the CAT reporter gene from the human immunodeficiency virus 1 long terminal repeat (HIV-1 LTR) sequences was stimulated by cisplatin in rat and human fibroblasts by 22- and 2.2-fold, respectively.[133,134] Carboplatin, a cisplatin analogue (Figure 1), does not show this effect, due to differences in the kinetics of hydration of the two compounds.[135]

The effects of cisplatin and *trans*-DDP on transcription were investigated by transfecting a plasmid containing the $\beta$-galactosidase reporter gene that had been modified by the compounds into human and hamster cell lines.[136] The level of transcription of the *trans*-DDP-modified plasmid was 2−3-fold higher

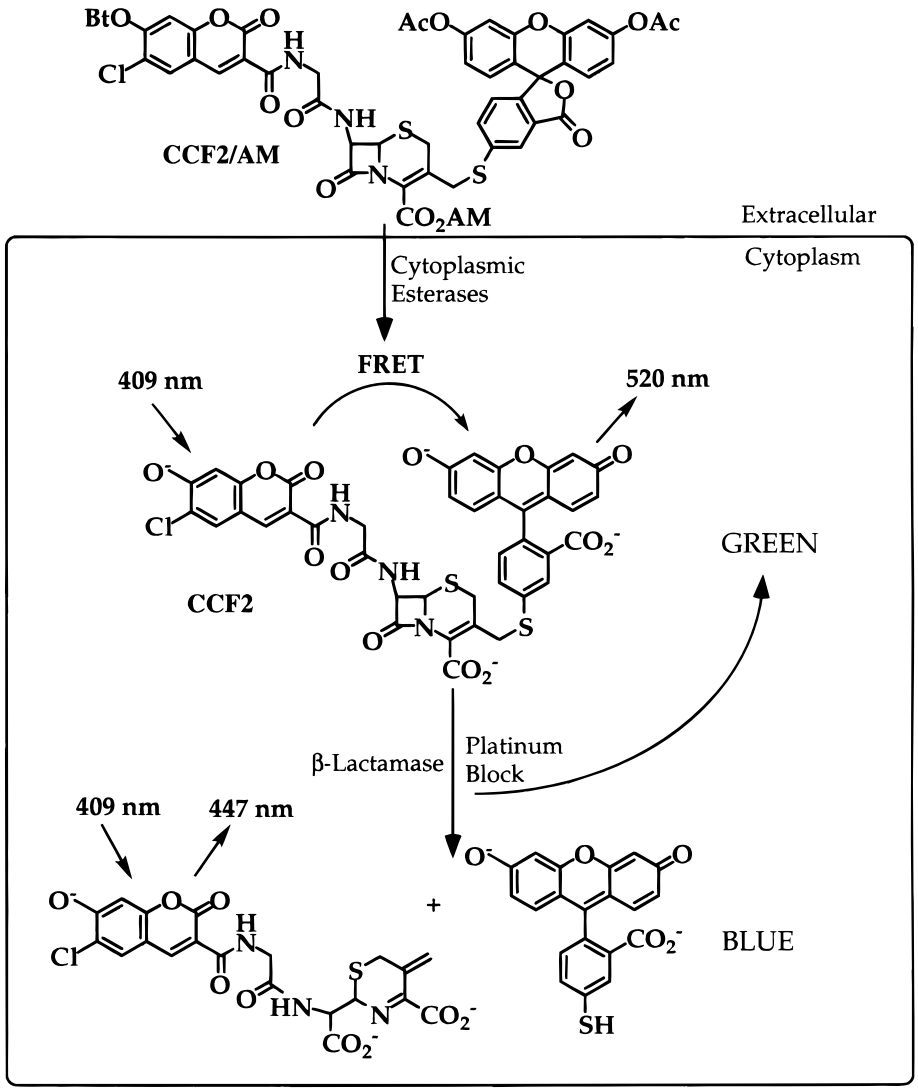


**Figure 13.** Uptake and the conversion of CCF2/AM to CCF2 in cells. In the presence of $\beta$-lactamase, CCF2 is cleaved to produce blue fluorescence. Treatment of cells with cisplatin blocks expression of $\beta$-lactamase and prevents cleavage of CCF2, resulting in green fluorescence.

than with the cisplatin-modified probe. Experiments performed in nucleotide excision repair (NER) deficient cell lines showed the same results, suggesting that the difference between the two compounds was not a consequence of differential NER of the adducts. These studies revealed that a 4-fold higher level of *trans*-DDP adducts is needed to inhibit RNA synthesis to an extent comparable to that of the cisplatin adducts, and the bypass efficiency of the RNA polymerase II was significantly higher (~60−76%) for the *trans*-DDP adducts compared to the cisplatin adducts (~0−16%). Other experiments examined the effect of cisplatin on hormone induced transcription from the mouse mammary tumor virus (MMTV) promoter which was stably incorporated into murine tumor cells.[137] Cisplatin inhibited the expression of the MMTV promoter in this system, presumably by altering the chromatin remodeling and loading of transcription factors that occur with the response of this promoter. *trans*-DDP did not similarly inhibit transcription. In additional studies where the MMTV promoter was transiently transfected into the cells, cisplatin blocked the binding of transcription factor NF1. The results of these experiments suggest that cisplatin may affect transcription by blocking transcription factors from binding and altering chromatin structure.

The ability of cisplatin to alter the normal binding of transcription factors has also been observed in work using a reconstituted system for measuring ribosomal RNA (rRNA) synthesis.[138] When pBR322 plasmid modified with cisplatin was added to this system, rRNA transcription was inhibited. This inhibition was correlated with the removal of the transcription factor, human upstream binding factor (hUBF), from its normal binding site on the rRNA promoter to the cisplatin-modified pBR322 plasmid. hUBF is a member of the HMG-domain family of proteins that bind specifically to cisplatin-modified DNA. This topic is addressed in more detail below.

The ability of cisplatin to inhibit transcription has recently been used as a method to screen rapidly for the potential antitumor activity of various platinum compounds. A HeLa Tet-on cell line was stably transfected with the enhanced green fluorescent protein (EGFP) under the control of the tetracycline-responsive element (TRE).[139] Treatment of these cells with cisplatin causes a dose-dependent decrease in

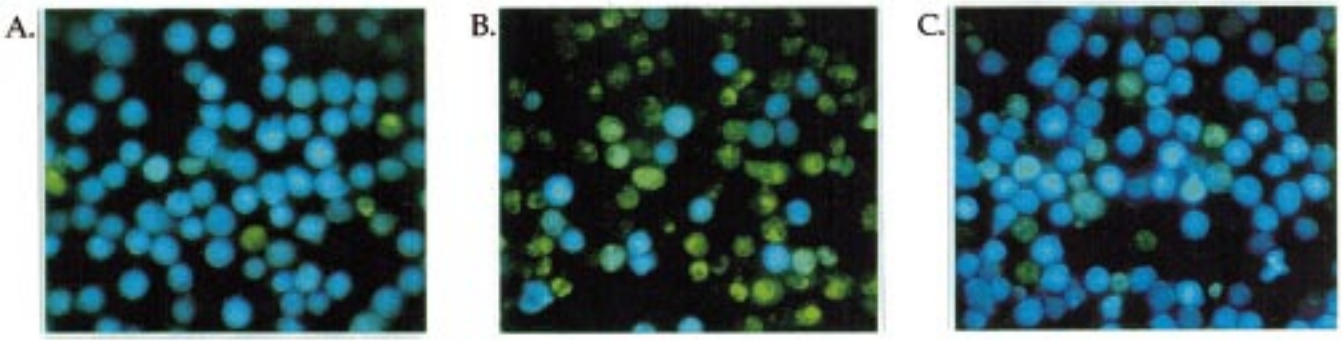


**Figure 14.** Results of the β-lactamase-CCF2/AM assay (see Figure 13). Panel A depicts Jurkat cells expressing β-lactamase treated with CCF2/AM. Panel B depicts Jurkat cells treated with cisplatin and CCF2/AM. Cisplatin treatment inhibits the expression of β-lactamase and causes the cells to have green fluorescence. Panel C depicts Jurkat cells treated with *trans*-DDP. This compound does not affect expression of β-lactamase, and the cells emit blue fluorescence. Reprinted with permission from ref 139. Copyright 1999 Elsevier Science Ltd.

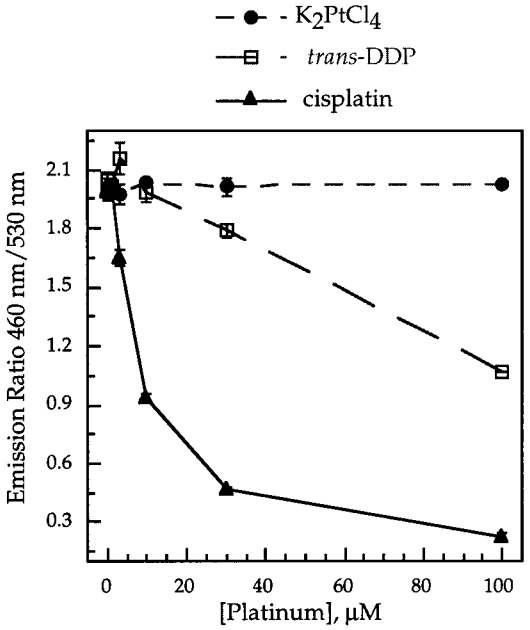


**Figure 15.** The effect of different platinum compounds on the expression of β-lactamase in the Jurkat cell assay (Figures 13 and 14) can be measured by examining the ratio of blue and green fluorescence in cells. This plot depicts the blue to green emission ratio for a few platinum compounds and shows cisplatin to be the most effective at inhibiting β-lactamase expression. Reprinted with permission from ref 139. Copyright 1999 Elsevier Science Ltd.

EGFP expression, presumably due to transcription inhibition, which can be monitored by using the fluorescent signal. A number of biologically active and inactive platinum compounds were screened with this assay. The results (Figure 12) show a striking correlation between the $LC_{50}$, the concentration of compound where 50% of the cells are dead, and the $IC_{50}$, the concentration of compound where gene expression is 50% of that of the control, demonstrating the potential utility of this mechanism-based screen. A similar assay uses the gene for β-lactamase stably transfected into a Jurkat cell line as a reporter. In this system, a fluorescent compound, CCF2-AM, is introduced into cells and converted into CCF2 by intracellular esterases (Figure 13).[140] In the presence of β-lactamase, CCF2 is cleaved and emits blue rather than green light. Cells treated with cisplatin have reduced expression of β-lactamase and revert to green (Figure 14). By measuring the emission ratio of blue and green light, the inhibition of β-lactamase can be quanitated for a number of different platinum compounds (Figure 15). Both of these assays have the advantage of being rapid compared to cytotoxicity assays and allow for convenient monitoring of the transcription inhibition through fluorescent signal changes. In addition, they highlight how understanding aspects of the cisplatin anticancer mechanism, such as transcription inhibition, can lead to advances in the methodologies used to develop new platinum anticancer drugs.

## C. Cisplatin, Telomeres, and Telomerase

The telomeric regions of DNA represent a very appealing target for cisplatin and may interfere with normal DNA function. Telomeres occur at the ends of eukaryotic chromosomes and consist of a tandem, G-rich repeat sequence. In humans, the sequence is 5′-TTAGGG-3′.[141,142] Part of their function is to protect the ends of the chromosomes from degradation and to ensure that the genetic information is properly inherited at each cell division.[143] During one cell division, telomeres are shortened by 50−200 bp.[144−146] When they become critically shortened, cells become senescent and die. One way cells can become immortalized is to counteract the shortening of the telomeres with the ribonucleoprotein telomerase. Telomerase synthesizes these repeat sequences at the ends of chromosomes and is postulated to play a role in the growth of malignant tumors.[147−149] Since the telomere-repeat sequences contain many guanosine residues, they represent a promising potential target for cisplatin.

Telomere loss in HeLa cells treated with cisplatin has been studied recently.[150] The results of these experiments demonstrated that telomeres in the cisplatin-treated cells were shortened and degraded. At low doses of cisplatin, telomere loss was sufficient to cause lethal damage in ∼61% of the cells. In another study, cisplatin inhibited telomerase activity in testicular cancer cells, whereas other DNA−damaging agents such as bleomycin and *trans*-DDP had no effect.[151] Cisplatin has the potential not only to interact with the G-rich telomere region of the chromosome but might bind to the RNA or protein component of telomerase. It could also effect telom-

erase expression. The results of the studies described above suggest that cisplatin can interfere with the normal functions of telomeres and telomerase, indicating a potential role in the anticancer mechanism. Much more work is required, however, to evaluate this possibility.

### D. DNA Damage and Apoptosis

Studies that demonstrated the ability of cisplatin to arrest murine leukemia L1210 and Chinese hamster ovary cells in $G_2$ phase led not only to the conclusion that cisplatin could block transcription but also provided information about the mechanism of cell death.[124,125] Cells treated with low concentrations of cisplatin recovered from $G_2$ arrest, whereas cells treated with higher concentrations of the drug had only a limited number of survivors.[124] The appearance of DNA double-strand breaks was the first detectable sign of cell death in these experiments, with the accumulation of debris on the flow cytometer and loss of trypan blue exclusion occurring later. Upon further investigation, the DNA double-strand breaks in L1210/0 cells were located in the nucleosome spacer region of chromatin DNA. These breaks appeared as "nucleosome ladders" in gel electrophoresis studies, identifying the mechanism of cell death to be apoptosis.[152]

Apoptosis, or "programmed cell death," is characterized by cell volume reduction, convolution and blebbing of the cell surface, chromatin condensation with activation of an endogenous endonuclease, recognition by phagocytic cells, and dependence on active protein synthesis.[153] The process occurs during embryonic development, metamorphosis, and general cell turnover.[154] As mentioned above, detection of nucleosome ladders in the L1210/0 cells was the first clue that cisplatin treatment induced cell death by apoptosis. Cell shrinkage and surface blebbing were also observed in that study.[152] Similar DNA fragmentation and cell shrinkage was seen with Chinese hamster ovary cells.[155] Treatment of cells with cycloheximide inhibited DNA fragmentation and cell death, consistent with new protein synthesis being required for the process to occur. Taken together, these results provided evidence that cisplatin can induce cells to undergo apoptosis and exposed a mechanism by which drug treatment kills cells.

## *IV. How Cells Process Platinated DNA*

Not only is it important to understand how cisplatin inhibits normal gene function, but active cellular processing of cisplatin-modified DNA also contributes to the mechanism of action of this drug. Understanding how cells respond to cisplatin DNA damage may also provide the knowledge required for improved platinum chemotherapy.

### A. Repair of Cisplatin–DNA Adducts

Studies that provided evidence for DNA as the primary target of cisplatin in cells also indicated how cisplatin−DNA adducts were processed. In particular, the cells deficient in DNA repair were much more sensitive than cells proficient in repair.[38−44] The increased sensitivity of the repair mutants implied that DNA was the target of the drug. This effect was not observed with *trans*-DDP.[42] In one study both nucleotide excision and recombination repair mutants were examined, suggesting that these pathways might be important in removing platinum adducts from DNA.[38] The ability of cells to modulate cisplatin toxicity through repair mechanisms indicated that this function might be important in the cellular processing of platinated DNA. The role of DNA repair in the cisplatin mechanism has been reviewed recently.[156,157]

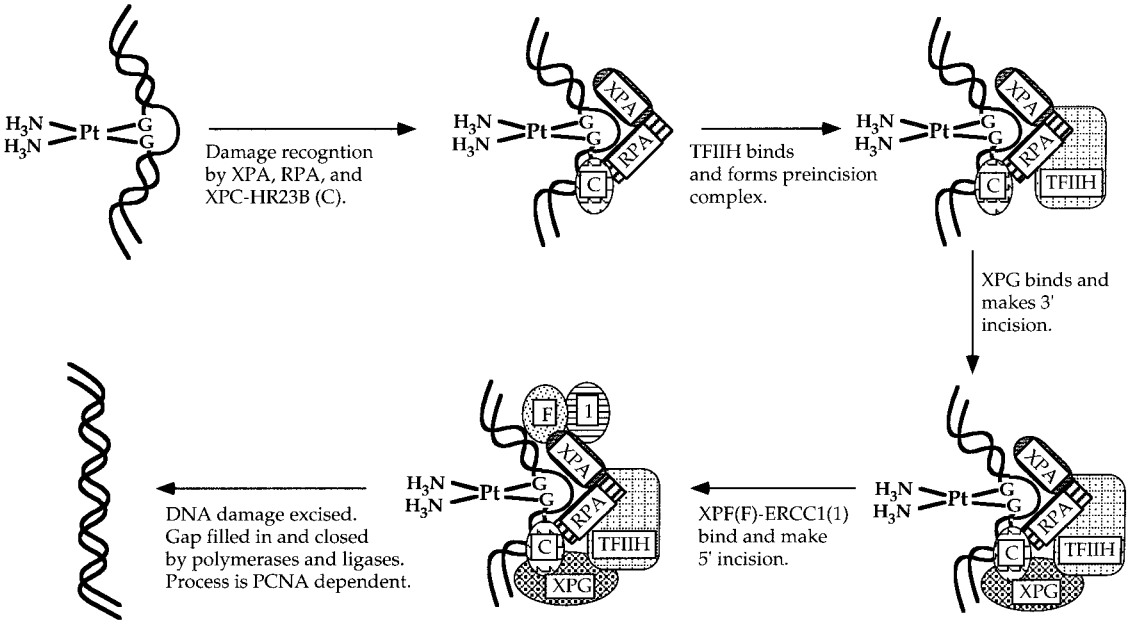


**Figure 16.** A schematic diagram of nucleotide excision repair of a cisplatin−DNA cross-link. The DNA damage is recognized by XPA, RPA, and XPC−HR23B (C). TFIIH binds forming a preincision complex. XPG makes the 3′-incision, and the 5′-incision is made by XPF−ERCC1 ((F) and (1), respectively). Once the damage is excised, the DNA is filled in by polymerases and ligases in a PCNA dependent process.

Cisplatin−DNA adducts are repaired in cells primarily through the nucleotide excision repair (NER) pathway.[158] This process involves many proteins and is used to repair a variety of DNA lesions, including damage caused by UV radiation. In eukaryotes, the DNA damage is excised as a ∼24−32-mer polynucleotide fragment,[159] whereas in prokaryotes the damage is removed as a ∼12−13-mer oligonucleotide.[160] The genetically inherited human disease xeroderma pigmentosum (XP) results from defects in NER.[159,161] Because of the inability to perform nucleotide excision repair, individuals with XP are extremely sensitive to UV radiation and have a predisposition toward skin cancer. XP has seven different genetic complementation groups, XP-A through XP-G, and a variant form, XP-V. The XP-A through XP-G groups are each deficient in a different component of the excision repair pathway. XP cells have an increased sensitivity to cisplatin treatment, providing further evidence that this pathway is important in the cellular processing of the drug.[162−164]

The mechanism of nucleotide excision repair is well-characterized (Figure 16), due in part to the availability of the different XP complementation groups (for recent reviews see refs 159−161). The first step involves recognition of DNA damage. The proteins XPA, RPA, and XPC have all been implicated in this process. These proteins bind to damaged DNA, but the order of their participation in the excision repair process is controversial. The XPC−HR23B protein complex was identified as the initial damage recognition protein in experiments that examined the rate of nucleotide excision repair while varying the order of addition of the proteins.[165] Similar types of experiments have produced contradictory results, indicating that it is an XPA−RPA complex that first recognizes DNA damage.[166] After the damage recognition step, TFIIH is recruited, forming a preincision complex with XPA, RPA, and XPC-HR23B. XPG binds and makes an incision spaced by 2−10 nucleotides to the 3′-side of the damage.[160] Very soon thereafter, the XPF−ERCC1 complex binds and makes a second cut 20−26 nucleotides to the 5′-side of the damage.[160] Once the piece of damaged DNA has been excised, the proteins dissociate, and the gap is filled in and closed by DNA polymerases and ligases. This repair synthesis step depends on proliferating cell nuclear antigen (PCNA) and is carried out by DNA polymerases $\delta$ and $\epsilon$. Several of the proteins involved in NER bind specifically to cisplatin-damaged DNA, a topic that will be discussed below.

As the effects of platinum compounds on DNA replication were being investigated, it was noted that cisplatin adducts accumulated on DNA continuously, whereas adducts of *trans*-DDP reached a maximum after a certain incubation period and decreased dramatically thereafter.[118] This effect was attributed to differential repair of the adducts rather than differential uptake of the compounds. The repair of cisplatin adducts has been studied more directly by using reconstituted repair systems. Nucleotide excision repair in *E. coli* has been extensively characterized. The activity results from three subunits, UvrA, UvrB, and UvrC, together referred to as the ABC excinuclease,[160] which excises *cis*-GG adducts from DNA restriction fragments modified with cisplatin.[42] DNA modified with *trans*-DDP produced a nonspecific cutting pattern, similar to that of an unmodified control. Extensions of this work demonstrated that the frequency of incisions by the ABC excinuclease was greater with cisplatin adducts than for *trans*-DDP adducts.[44] When plasmids damaged with the two compounds were transformed into various strains of repair proficient and deficient strains of *E. coli*, the uvrB gene was found to be essential for repair of cisplatin-damaged DNA, indicating NER to be the primary pathway for repair of these adducts.[44] The results of these experiments were consistent with the suggestion that *trans*-DDP adducts are repaired by a different cellular mechanism.

The development of an in vitro repair synthesis assay using mammalian cell extracts allowed further investigation of the repair of cisplatin−DNA adducts. Initial work demonstrated that plasmid DNA modified with either cisplatin or *trans*-DDP was repaired in this assay.[167] Cell extracts prepared from various XP cell lines did not exhibit repair synthesis, as would be expected owing to their deficiencies in NER.[167,168] Plasmids modified with *trans*-DDP were better repaired than cisplatin-modified plasmids when this assay was performed with HeLa and 293 cell extracts.[119] Repair synthesis was initially not observed with a plasmid containing a site-specific *cis*-GG adduct, suggesting that this adduct is poorly repaired in human cell extracts.[169] The repair synthesis activity could be detected for the *cis*-GG plasmid when it was preincubated with the *E. coli* UvrABC excinuclease, indicating that the human enzymes might not be able to make the necessary incisions on the damaged DNA.

When a different type of excision repair assay was used to study cisplatin adducts in vitro, the seeming inability of the *cis*-GG adduct to be repaired was not confirmed. Unlike the repair synthesis assay, which measures the incorporation of a radiolabel into damaged DNA compared to an undamaged control, the excision assay examines the release of the damaged ∼26−32 nucleotide oligomer. Studies using both human cell free extracts and a reconstituted excinuclease revealed the *cis*-GTG adduct to be more efficiently repaired than either the *cis*-AG or *cis*-GG adduct.[170,171] No repair was detected for a cisplatin interstrand cross-link. These results were subsequently confirmed with the repair synthesis assay, where the *cis*-GTG adduct was repaired ∼15−20-fold better than the 1,2-intrastrand adducts.[172] This repair profile had been observed previously for adducts of the cisplatin analogues [Pt(dach)$Cl_2$] and [Pt(en)$Cl_2$] with the ABC excinuclease system.[173] The excision repair assay has also been used to examine the role of cellular proteins in modulating the repair of cisplatin adducts, as will be discussed.[170,171]

The rate at which the cisplatin adducts are excised from DNA has also been investigated. Initial studies used various normal and repair-deficient human fibroblast cells to examine the rate of repair.[164] In normal and Fanconi's anemia (FA) fibroblasts, there

was a very fast initial removal of more than half the cisplatin adducts. In XP fibroblasts, however, this fast initial repair phase was absent, and there was a slow, gradual removal of the adducts. Later work using ABC excinuclease digestion demonstrated that cisplatin intrastrand adducts are removed faster from transcribed genes compared to nontranscribed genes and the overall genome.[174] Strand bias was also observed for these adducts, the intrastrand adducts being preferentially repaired from the transcribed strand.[175] These studies demonstrated that the cisplatin interstrand adducts are removed more efficiently from genes than intrastrand adducts, and there did not appear to be any preferential repair of the interstrand adducts from transcribed versus nontranscribed genes or DNA strands. These results for the cisplatin intrastrand adducts are consistent with the notion of transcription-coupled repair.[176]

There are other cellular mechanisms, besides nucleotide excision repair, that can affect the cytotoxicity of cisplatin adducts. *E. coli* cells having mutations in either recombination or mismatch repair showed enhanced sensitivity to cisplatin, suggesting a potential contribution of these pathways.[38,43] More recent studies have provided additional evidence for the participation of the mismatch repair system in processing cisplatin adducts (for a review of mismatch repair see ref 177). Human MutSα is a heterodimer of the proteins MSH2 and MSH6 (GTBP/p160) involved in mismatch recognition, and both MutSα and MSH2 bind with some selectivity to DNA containing the *cis*-GG adduct.[178−180] This preference was not observed for the *cis*-GTG adduct or for DNA adducts of $[Pt(dien)Cl]^+$.[178,179] hMSH2 is overexpressed in the testes and ovaries, two tumor types most effectively treated by cisplatin.[179] A loss of mismatch repair abilities was correlated with a 2.3-fold gain of cisplatin resistance in some cell lines.[177,181−186] Thus, whereas a larger body of evidence is required before mismatch repair can be proved to be important in the cisplatin anticancer mechanism, these initial results suggest that it may play a role in the cellular response to platinated DNA.

## B. Cellular Resistance to Cisplatin

One of the main reasons for failure of cisplatin treatment is resistance of tumors to the drug. Resistance to cisplatin can either be intrinsic to cells or acquired through exposure to the compound. Some tissues are inherently resistant to cisplatin and do not respond to treatment. Other types of malignancies, such as ovarian cancer, may respond initially to cisplatin treatment but can acquire resistance to the drug over time. Because of its clinical importance, the mechanism of cisplatin resistance has been studied extensively (for reviews of this topic, see refs 158 and 187−189). Three main activities have been identified as potential responses that modulate the resistance. These include changes in intracellular accumulation of the drug, increased production of intracellular thiols to modulate toxicity, and increased capability of cells to repair cisplatin−DNA damage. Many studies have been performed in attempts to understand resistance, but the results are often contradictory, as will be shown below. Resistance of cells to cisplatin appears to be a multifactorial cellular response that makes it difficult to understand the process fully.

Cisplatin-resistant cell lines can be made through repeated exposure to the drug. Comparisons between the parental and resistant lines provide information about the mechanism of resistance. The level of platinum accumulation has been examined in several different cell lines having acquired cisplatin resistance, including human head and neck squamous cell carcinoma, human small cell lung carcinoma, murine leukemia L1210, human T lymphocytes, human ovarian carcinoma, and human testicular nonseminomatous germ cells.[190−203] With the human head and neck squamous carcinoma cell line, the resistant cells had a reduced capacity to take up cisplatin, whereas release of the drug was similar to the parental cell line.[190] Studies in murine leukemia L1210 cells found varying results. Some cisplatin-resistant L1210 cells displayed a ∼40−50% reduction in drug accumulation.[191,194] In other studies, uptake was markedly reduced (36−60% and 3-fold in refs 26 and 192, respectively), while no difference in efflux of the drug was observed between the sensitive and resistant cell lines.[26,192] The results of other experiments, however, demonstrated no significant differences in the amount of drug taken up by cell nuclei, and the amount of cisplatin bound to DNA was similar in sensitive and resistant L1210 cells.[202] Discrepancies in platinum accumulation were also observed in ovarian cell lines. The level of platinum accumulation was decreased by ∼50% in one study,[193] and another showed a decrease that correlated with resistance.[196] Varying selection conditions produced differing results; one cisplatin resistant cell line showed a ∼50% decrease in cisplatin accumulation, while another had the same amount of accumulation as the sensitive parental cell line.[195] In a different study, the rate of drug accumulation was similar to parental cells, but the resistant cell line was more efficient at effluxing cisplatin.[198] Human epidermoid KB carcinoma cells have enhanced efflux of cisplatin,[204] and intracellular cisplatin accumulation was ∼1.6-fold greater in a sensitive testicular nonseminomatous germ cell line.[201] For small cell lung carcinoma cells, no difference was found in platinum content between sensitive and resistant cells after correction for cellular protein and volume differences.[203] Taken together, these studies suggest that, whereas decreased cellular accumulation due to either reduced uptake or enhanced efflux may play a role in cisplatin resistance, it is clearly not a consistent feature of this phenotype.

Another cellular response that can modulate cisplatin resistance is to increase the level of intracellular thiols that can react with and inactivate cisplatin. Glutathione (γ-glutamylcysteinylglycine, GSH) is the most abundant thiol in cells, found at concentrations of 0.5−10 mM.[158] This tripeptide is synthesized in a two-step pathway that is ATP-dependent. The enzyme γ-glutamylcysteine synthetase is involved in the first step and can be inhibited by D,L-buthionine-(*S*,*R*)-sulfoximine (BSO). The second step

uses the enzyme glutathione synthetase to complete the peptide synthesis. Many studies have been performed to determine whether an increase in glutathione levels causes cisplatin resistance in different cell lines.

In a resistant human small cell lung carcinoma line, the amount of GSH and the total amount of sulfhydryl compounds was increased compared to the parental, sensitive line.[203] The results in ovarian carcinoma cell lines were variable. An increase in GSH levels in resistant cell lines occurred in some cases[205−207] and was correlated with the sensitivity of different ovarian cell lines.[208] In a different study, GSH levels were not increased in one cell line at low levels of cisplatin resistance (2−3-fold) but were elevated by 30% at higher (9-fold) levels of resistance.[195] A different cell line in that study demonstrated a 2.3-fold increased level of GSH at a 13-fold increase in cisplatin resistance. For both of these cell lines, the resistance to cisplatin could be partially reversed through depletion of GSH by addition of BSO.

The level of GSH was found to fluctuate in some ovarian cell lines over time.[209] In another study, resistant ovarian carcinoma cells had similar levels of GSH as the parental line, and the addition of BSO did not increase the sensitivity of the cells to cisplatin.[210] Two studies of cisplatin resistant murine leukemia L1210 cells demonstrated increased levels of GSH.[191,211] The addition of BSO to these cell lines caused different responses, with one cell line increasing its sensitivity to cisplatin,[211] whereas BSO had no effect on the other.[191] Finally, a study in human colon carcinoma cells noted about a 3-fold increase in GSH in cisplatin resistant cells,[212] but studies with human testicular nonseminomatous germ cells observed no difference in GSH levels between sensitive and resistant cells.[201] The sum of the evidence from these investigations indicates that an increase in GSH levels is likely to be a factor involved in cisplatin resistance, but not an absolute requirement.

Another intracellular thiol that could play a role in cisplatin resistance is metallothionein. This protein consists of ~61−62 amino acids, 20 of which are cysteine, and is presumed to take part in the detoxification of heavy metal ions in cells.[158] In a study of a variety of tumor cell lines with acquired cisplatin resistance, metallothionein was overexpressed.[213] A modest enhancement in metallothionein levels was also observed in a resistant human testicular nonseminomatous germ cell line.[201] Studies in human ovarian cancer cells showed variable expression of metallothionein, with different cell lines exhibiting different levels of resistance.[214] This work detected no relationship between metallothionein expression and cisplatin resistance. Studies examining the overall levels of total sulfhydryl content of resistant cells produced varied results. A human head and neck squamous carcinoma line showed a 2-fold increase in protein sulfhydryl content,[190] while no increase was observed in a study with murine L1210 cells.[192] Thus, although there is not as much evidence for metallothionein participation in cisplatin resistance as for GSH, it is possible that this protein may mediate the toxicity of this drug.

The final potential mechanism for cisplatin resistance to be discussed here is the ability of resistant cells to undergo enhanced DNA repair, which has been extensively investigated. Numerous experiments have revealed enhanced repair in cisplatin-resistant cells, including studies in human ovarian cancer cell lines,[197,198,206,215−218] murine leukemia L1210 cells,[194] Chinese hamster ovary cells,[219] and cells from a human malignant glioma.[220] Whereas the ability of resistant cell lines to undergo enhanced repair appears to be a common theme in these various cell lines, increased repair did not always correlate with the level of cisplatin resistance.[194,216,219] Often enhanced repair was suggested to be part of the resistance mechanism in conjunction with some other cellular response.[194,198,218,220] Differential DNA repair has also been noted in samples taken from biopsies of patients with ovarian carcinoma who were not treated with cisplatin.[221] There is as much as a 10-fold difference in the ability of these ovarian cells to repair DNA damage, suggesting that some tumors are intrinsically poor in repairing cisplatin lesions. A similar observation was made when examining a testicular nonseminomatous germ cell line.[201] Here, the difference in repair observed between cisplatin-sensitive and -resistant cell lines was attributed to an inherent defect in the ability of the parental cells to repair platinum adducts from their DNA. Very recent work has provided evidence in support of this idea by demonstrating that the levels of the excision repair proteins XPA and the ERCC1−XPF complex are reduced in human testis tumor cell lines.[222] These results suggest a basis for the organotropic specificity of cisplatin. In addition to this work, some investigations have shown that DNA repair proteins, such as XPA, XPE, ERCC1, and DNA polymerase $\beta$, can be overexpressed in resistant cell lines.[223−226] Taken together, these results suggest that enhanced DNA repair is an important aspect of cisplatin resistance. However, the studies examining increased cellular accumulation and intracellular thiol levels imply that it is unlikely that enhanced DNA repair alone is sufficient to effect cisplatin resistance.

The pathways of increased intracellular platinum accumulation, elevated levels of intracellular thiols, and enhanced DNA repair have been presumed to be important in cisplatin resistance, and many studies have examined their potential roles in this process. Other cellular responses may also be important in resistance. An interesting study was performed with EMT-6 murine mammary tumors that were made resistant to cisplatin in vivo.[227] These tumor cells were then established in culture. Surprisingly, the cultured cells showed no signs of resistance, but resistance was re-established after implanting the cultured cells into mice. The results of this experiment suggest that some mechanisms of acquired resistance only occur in vivo. The loss of mismatch repair activity has also been observed in cisplatin-resistant cells.[177,181−185] It was not determined exactly how loss of mismatch repair could contribute to cisplatin resistance, but it may prevent a futile cycle of DNA repair which results in apo-

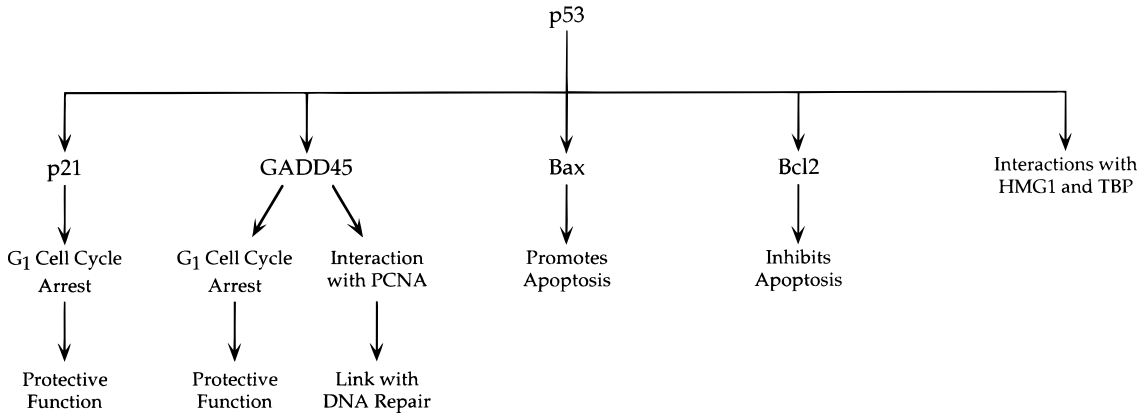


**Figure 17.** A representation of some of the proteins and genes that may be influenced by p53 in a cellular response to cisplatin. The implications for cellular function are also listed.

ptosis, as discussed below.[182] Loss of p53 function, which controls many cellular responses, has also been linked to cisplatin resistance.[228−230] This topic is also discussed in more detail below. Another potential resistance mechanism is the increased ability of the cell to tolerate DNA damage.[189] It is not clear exactly how cells might gain tolerance to DNA damage, but some possibilities include the increased ability to bypass DNA adducts during replication or changes in the amount of DNA damage necessary to signal apoptosis. Studies in human ovarian carcinoma cells revealed a 2.3−4.5-fold increase in the replicative bypass ability of cisplatin-resistant cells.[231] Similar work discovered resistant murine leukemia cells to have an increased bypass ability as well.[232] Studies with ovarian cancer cells attributed part of the resistance mechanism to increased cellular tolerance of DNA damage,[218,233] and in one of these studies, it was hypothesized that the cisplatin-resistant cells required higher levels of DNA damage to undergo apoptosis.[233] Thus, from the experiments presented to date, the mechanism of cisplatin resistance appears to be complex and multifactorial. These studies provide insight into some of the possibilities, but more work will need to be done before this clinically important problem can be solved.

## C. Effect on p53 and Cellular Response

The p53 protein has been well-studied, in part because of the high frequencies of mutations in the p53 gene in human cancers (for reviews, see refs 234−236). p53 is a transcription factor that is involved in the regulation of many genes including p21, MDM2, GADD45, cyclin G, and Bax.[235] The activation of p53 due to DNA damage or other cellular stresses can result in cell cycle arrest and apoptosis. This response helps to maintain genomic stability, the loss of which can result in the development of cancer.[236] Disruption of normal p53 function occurs in about half of human cancers, but testicular tumors, which are most successfully cured by cisplatin, do not generally contain mutated p53 genes.[235,236]

The role of p53 in the cisplatin mechanism has been examined (for a review, see ref 157). As mentioned above, loss of p53 function confers resistance in some human ovarian cancer cell lines, presumably by interfering with the regulation of apoptosis.[228−230] The conference of resistance by loss of p53 depends on the genetic context of the cell line.[228] For example, a study in breast cancer MCF-7 cells, which do not readily undergo apoptosis following cell damage, demonstrated that disruption of p53 function sensitized the cells to cisplatin.[237] Overexpression of a temperature-sensitive mutant form of p53 increased resistance in an ovarian cell line, and p53 was overexpressed during the development of resistance.[238] The mechanism by which overexpression of p53 would confer resistance is unclear, but it may interfere with DNA repair capabilities or somehow enhance cellular tolerance to DNA damage.[238] Finally, some studies suggest that cisplatin cytotoxicity is not influenced by p53. The presence of normal or mutant p53 in nine ovarian cancer cell lines did not affect the cisplatin cytotoxicity,[239] and p53 mutation in mouse testicular teratocarcinoma cells did not confer resistance to those cells.[240] Thus, the results of these selected studies show that, as with the other mechanisms of cellular resistance, p53 function may only be one of many factors that modulate cisplatin sensitivity, and the effects may be different for various cell types.

Because p53 controls the transcription of several genes, it could affect cellular responses to cisplatin indirectly through its regulation of other proteins (Figure 17). One of the proteins under the control of p53 is p21 (WAF1/Cip1). The p21 protein is involved in $G_1$ cell cycle arrest.[234,235] Overexpression of p21 in glioblastoma cells conferred resistance to cisplatin.[241] These cells showed enhanced repair of DNA damaged with the alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea, suggesting that the presence of p21 arrests the cell cycle and allows repair of DNA damage, protecting the cell from apoptosis. The protective function of p21 was demonstrated in another study where human colon cancer cells containing a disrupted p21 gene were more sensitive to cisplatin.[242] The enhanced sensitivity was also seen in embryonic fibroblasts from p21 knockout mice.[242] The loss of p21 function in colon cancer cells impaired their ability to repair cisplatin-damaged CAT-reporter plasmids, suggesting that the p21 protection is related to DNA repair.

Aside from the p21 pathway, p53 could affect DNA repair through GADD45, another gene product that

it activates. Production of GADD45 is induced by DNA damage and can also lead to $G_1$ cell cycle arrest.[234,235] GADD45 binds to the repair factor PCNA, potentially affecting nucleotide excision repair.[234,235] Disruption of normal p53 function reduces repair of UV-damaged DNA, indicating that p53 or its gene products can affect the nucleotide excision repair pathway.[243] Later work demonstrated a link to GADD45, because cells where expression of GADD45 was blocked by antisense vectors exhibited altered levels of DNA repair, and blocking GADD45 expression increased the sensitivity of cells to cisplatin.[244] An additional link between p53 and NER has recently been hypothesized to occur through the XP gene, p48.[245] The p48 gene is essential for binding UV-damaged DNA and is disrupted in the subset of XPE cells that lack this activity. p48 mRNA levels vary with p53 expression, being more prevalent in p53 normal cells compared to p53 mutants and increasing with raised p53 levels following UV irradiation. The ability of p53 to control levels of p48 suggests another pathway by which this protein could affect DNA repair.

p53 not only regulates genes that control the cell cycle and interfere with DNA repair, it also affects genes involved in the apoptotic response. p53 can stimulate the production of Bax, which promotes apoptosis, and it can also repress the production of Bcl-2, which inhibits apoptosis.[234] The relative ratios and dimerization of such proteins is suggested to be important in regulating apoptosis.[246,247] Both Bcl-2 and Bax have multiple isoforms, produced by alternative RNA splicing, that can have different functions and cellular localizations.[247] Cisplatin resistant ovarian cancer cells have reduced levels of Bax mRNA, consistent with an ability to inhibit apoptosis.[230] In other work, a 21 kDa Bax protein was upregulated in p53 functional cells after exposure to cisplatin, but levels of Bcl-2 and a 24 kDa Bax protein were unchanged.[248] The 21 kDa Bax was not expressed constitutively in these cells, and it is possible that the 21 kDa protein that appears after drug treatment could be a cleavage product of the 24 kDa Bax protein.[248] Alternatively, treatment with cisplatin could produce cellular conditions that favor the 21 kDa isoform of Bax over the 24 kDa form. Overexpression of Bcl-2 confers cisplatin resistance to cells, presumably through its ability to inhibit apoptosis.[238,249,250]

The involvement of p53 in the cellular processing of cisplatin may include interaction with other proteins in addition to its ability to regulate gene transcription. p53 interacts with high mobility group protein 1 (HMG1) and the TATA binding protein (TBP).[251,252] Both of these proteins bind specifically to cisplatin-modified DNA, as discussed below. The presence of cisplatin−DNA adducts in cells could affect the interactions of these proteins and alter their natural functions. At present, there is no evidence to suggest that these protein interactions may participate in the cisplatin mechanism, but it is intriguing that proteins which specifically bind cisplatin-damaged DNA also have the potential to interact with the important transcriptional regulator p53.

## V. Cellular Proteins that Bind to Cisplatin–DNA Adducts

Thus far, the effect of cisplatin on DNA function and some consequential cellular responses have been examined. The ability of cisplatin to inhibit replication and transcription is not absolute, suggesting that there must be other factors involved which lead to cell death. Identifying these factors and understanding how they cause cell death is crucial to unraveling the mechanism of action of cisplatin. Cellular proteins are likely to be involved in this process, and a search for proteins that can specifically bind to cisplatin-modified DNA was therefore undertaken. To date, many such proteins have been identified that share this property (Table 3), and mechanisms have been proposed for how they may effect a cytotoxic response (for a recent review, see ref 253).

### A. Repair Proteins

One class of proteins that bind cisplatin-modified DNA are involved in repair, which is not surprising considering that cisplatin−DNA adducts are processed by the NER system. One of the first such proteins to be identified was XPE (UV-DRP),[224,254] the role of which is still unknown. It consists of two subunits of 127 and 48 kDa molecular mass and binds damaged DNA.[159] XPE is not a component of the core nucleotide excision repair system, but may play an accessory role.[159] Cisplatin-resistant human tumor cell lines overexpress XPE and show enhanced DNA repair capabilities.[224] XPE can also be induced by cisplatin treatment,[255] the level of which correlated with cisplatin resistance and required de novo RNA and protein synthesis. Induction of XPE was attributed either to formation of platinum−DNA adducts or to inhibition of DNA replication.

The proteins XPA, RPA (single-stranded binding protein, SSB), and XPC−HR23B have been reported to recognize DNA damage in the nucleotide excision repair pathway (Figure 16). XPA consists of 273 amino acids, has a molecular weight of ~31 kDa, and contains a zinc finger motif.[256,257] RPA is made up of three 70, 32, and 14 kDa subunits.[258] These two proteins both bind individually and specifically to cisplatin-modified DNA,[256,257,259−261] and they also cooperatively bind cisplatin-modified DNA.[262] The DNA binding domain of XPA has been determined.[263] This truncated, 122 amino acid XPA fragment retains the ability to bind specifically to cisplatin-damaged DNA. RPA binds to DNA containing a *cis*-GNG adduct 1.5−2-fold better than to DNA containing a *cis*-GG adduct.[261] It has been hypothesized that RPA helps to denature the damaged DNA duplex and subsequently binds the resulting single-stranded DNA. The ability of XPC−HR23B to bind preferentially to cisplatin-damaged DNA has been demonstrated through coimmunoprecipitation assays.[165] Another protein involved in nucleotide excision repair is ERCC1. This protein forms a complex with XPF and helps to make an incision on the 5′-side of the

**Table 3. Proteins that Bind to Cisplatin-Modified DNA**

| protein | function | $K_d$[a] | specificity ($\rho$)[a] | ref |
|---|---|---|---|---|
| | Repair Proteins | | | |
| XPE (UV-DRP) | potential accessory role in nucleotide excision repair | nd | nd | 224,254,255 |
| XPA (xeroderma pigmentosum A complementing protein) | damage recognition protein in nucleotide excision repair | nd | nd | 256,257,263 |
| RPA (replication protein A, single stranded binding protein, SSB) | damage recognition protein in nucleotide excision repair | nd | 4−6-fold | 259,260 |
| XPC-HR23B | damage recognition proteins in nucleotide excision repair | nd | nd | 165 |
| MutSα, MSH2 | recognition component in mismatch repair | 67 nM | nd | 178−180 |
| Ku autoantigen (DNA−PK) | takes part in V(D)J recombination and double-strand break repair | 0.11 nM | nd | 265,266 |
| DNA photolyase | repair of cyclobutane pyrimidine dimers | 50 nM | nd | 267,268 |
| T4 endonuclease VII | cleaves branched DNA structures | nd | nd | 269 |
| | HMG-Domain Proteins | | | |
| human SSRP1 | involved in transcription elongation | nd | nd | 271−273,312 |
| T160 | involved in V(D)J recombination | nd | nd | 274 |
| *Drosophila* SSRP1 | unknown | nd | nd | 275 |
| HMG1 | unknown | 0.3−370 nM | 100-fold | 276,280−282 |
| HMG2 | unknown | 0.2 nM | nd | 276,281 |
| Ixr1 | transcription factor that regulates Cox5b promoter | 250 nM | 10-fold | 283,285 |
| HMG-D | *Drosophila* homologue of HMG1 | 200 nM | 2−3-fold | 286 |
| mtTFA | mitochondrial transcription factor | ∼100 nM | nd | 287 |
| hUBF | ribosomal RNA transcription factor | 60 pM | nd | 288 |
| tsHMG | unknown | 24 nM | 230-fold | 289 |
| SRY | sex determining factor | 120 nM | 20-fold | 290 |
| Cmb1 | mismatch recognition factor | nd | nd | 291 |
| HMG1 domain A | one of two DNA binding domains of HMG1 | 0.2−517 nM[b] | 2−1000-fold[b] | 293,299 |
| HMG1 domain B | one of two DNA binding domains of HMG1 | 48−1300 nM[b] | 3−4-fold[b] | 287,292,293 |
| tsHMG domain A | one of two DNA binding domain from tsHMG | 300 nM | 20-fold | 289 |
| human SRY domain | DNA binding domain from SRY | 4 nM | ∼5-fold | 290 |
| mouse SRY domain | DNA binding domain from SRY | 1 $\mu$M | nd | 287 |
| LEF-1 domain | DNA binding domain from LEF-1 | ∼100 nM | nd | 287 |
| | Transcription Factors Lacking an HMG Domain | | | |
| TATA binding protein | part of the basal transcription factor TFIID | nd | nd | 308,318 |
| YB-1 | transcription factor | nd | nd | 321 |
| | Architectural Proteins without an HMG Domain | | | |
| Histone H1 | linker histone | nd | nd | 324,325 |

[a] Values not determined are denoted as nd. [b] Dependent on flanking sequence content.[293]

DNA damage (Figure 16).[159] ERCC1 expression levels are elevated in some cisplatin resistant cells,[225] and the binding of XPA to damaged DNA is increased through interaction with ERCC1.[264] These results suggest that ERCC1 may participate with XPA in the recognition of damaged DNA.

Proteins involved in the mismatch repair pathway also bind with some specificity to cisplatin-modified DNA. The recognition component of the mismatch repair pathway, MutSα, is a heterodimer comprising MSH2 and MSH6 (GTBP/p160). Normally, this complex binds to single base mismatches, loops of one base, and loops of two bases.[180] Both MutSα and its MSH2 component bind to cisplatin-modified DNA.[178−180] The binding of MutSα was enhanced by the presence of a G:T mismatch at the guanine to the 3′-side of the 1,2-d(GpG) intrastrand cross-link.[180] Such a mismatch could occur if the damaged DNA had undergone replication.[184] It is interesting that MSH2 is overexpressed in testicular and ovarian tissues, which are most effectively treated by cisplatin.[179]

As mentioned above, mismatch repair deficiencies have been noticed in some,[181−185] but not all, cisplatin-resistant cells. In fact, it has been suggested that treatment with cisplatin selects for cells with a preexisting mismatch repair defect.[186] One possible explanation for how loss of this activity could confer resistance is that the mismatch repair pathway could recognize a cisplatin adduct in the template strand and attempt to repair the newly synthesized, non-platinated strand.[182] This activity would result in a futile cycle, because the mismatch could never be repaired as long as the cisplatin adduct persisted (Figure 18). The futile cycle of repair has been hypothesized to generate a signal for apoptosis, although proof of such an activity is lacking. In resistant cell lines, a defect in mismatch repair would not allow this signal to be generated, enhancing cell survival.

Human Ku autoantigen, a component of the DNA−dependent protein kinase (DNA−PK), takes part in V(D)J recombination and double-strand break repair and binds to DNA damaged by cisplatin.[265] DNA−PK is a heterotrimeric complex containing a dimeric DNA binding subunit, Ku, and a catalytic subunit, DNA−$PK_{cs}$.[266] Normally, Ku binds to DNA, and the Ku−DNA complex activates DNA−$PK_{cs}$ activity. The activated DNA−PK complex can phosphorylate a number of different proteins, including RPA, p53, and other transcription factors.[265,266] When Ku binds to

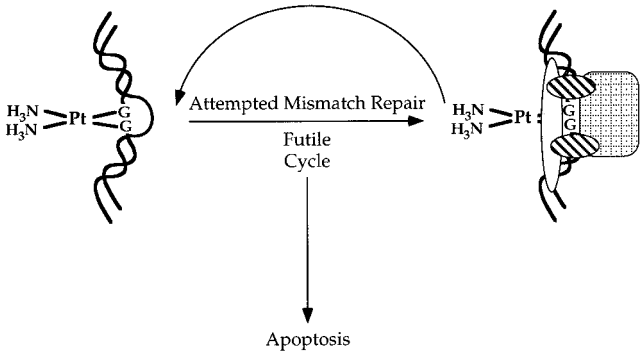


**Figure 18.** A schematic diagram of how mismatch repair activity could promote apoptosis. The mismatch repair proteins could recognize the cisplatin adduct as a mismatch and attempt to repair it. As long the cisplatin adduct persists, the mismatch cannot be repaired, resulting in a futile cycle that could signal apoptosis.

cisplatin-modified DNA, DNA−P$K_{cs}$ is not activated, potentially interfering with the regulation of transcription factors through phosphorylation.[265] The mechanism for this inhibition has been studied. Ku binds to cisplatin-modified DNA nonspecifically. The affinity for cisplatin-damaged DNA is similar to that for undamaged DNA, and the ability of the protein to bind the *cis*-GG, *cis*-AG, and *cis*-GNG adducts was also similar.[266] The degree of DNA−PK inhibition, however, varied with the nature of the cisplatin−DNA adduct. It was suggested that the different structures of the adducts interact differently with DNA−PK. This hypothesis was supported by the fact that the position of the platinum lesion on the DNA substrate also changed the degree of inhibition. This study concluded that the mechanism of DNA−PK inhibition was due to a large decrease in $V_{max}$ and a small increase in $K_M$.[266]

A DNA repair enzyme that binds to cisplatin-damaged DNA is photolyase. Its binding specificity has been demonstrated with both yeast and *E. coli* photolyase.[267,268] DNA photolyase is an enzyme that repairs cyclobutane pyrimidine dimers created by UV radiation. It occurs in *E. coli*, yeast, *Drosophila melanogaster*, fish, birds, and marsupial mammals, but has not been detected in humans.[267] In the yeast study, the binding of photolyase sensitized the cells to the drug, possibly by blocking nucleotide excision repair or by creating a signal for cell death.[267] The studies in *E. coli* showed the opposite effect, where the binding of photolyase stimulated the removal of the platinum lesion by a nucleotide excision repair pathway and made cells more resistant to cisplatin. The different effects in these two systems have not been explained, but may be due in part to differences in the nucleotide excision repair system between yeast and bacteria.

Finally, T4 endonuclease VII is an enzyme that cleaves branched DNA structures, including four-way DNA junctions.[269] This enzyme is required in T4 infection to resolve branchpoints created by DNA replication, and it is present in many different organisms, including mammals.[269] This enzyme binds and cleaves *cis*-GG and *cis*-AG adducts specifically.[269] It has no effect on a *trans*-DDP 1,3-d(GpTpG) cross-link. T4 endonuclease VII cleaves DNA containing cisplatin interstrand cross-links preferentially over DNA with interstrand cross-links of *trans*-DDP.[270] It has been suggested that the specific structural distortions of the cisplatin adducts cause the recognition by this enzyme,[269] however, the ability of this enzyme to recognize both 1,2-interstrand adducts and interstrand cross-links casts doubt on this hypothesis.

## B. HMG-Domain Proteins

### *1. Nature of Binding to Platinated DNA*

High mobility group (HMG) domain proteins represent a large family of proteins that bind specifically to cisplatin-modified DNA. The discovery of this binding ability was made approximately 10 years ago during a search for cellular factors that could play a role in mediating the cisplatin mechanism of action. Modified Western blots identified factors in HeLa cell extracts that bound preferentially to cisplatin-modified DNA.[271] This initial screen located species of ∼100 and 28 kDa that bound to double-stranded DNA modified with cisplatin or [Pt(en)$Cl_2$], but not to DNA modified with *trans*-DDP or [Pt(dien)Cl]Cl, both inactive compounds. Experiments performed using HeLa and hamster V79 cell extracts identified a protein of ∼91 kDa that bound to *cis*-GG and *cis*-AG adducts, but not to *cis*-GNG adducts or DNA modified by *trans*-DDP.[272] The presence of selective binding factors in cell extracts suggested that it would be possible to isolate cDNA clones encoding proteins with this ability. By screening of a human B-cell expression library, two such clones were obtained.[271] Northern blot analysis of one of these partial clones predicted that the molecular mass of the full-length protein would be 100 kDa. Further expression library screening with the partial clones led to the isolation of the full-length cDNA that predicted an ∼81 kDa protein. This factor was called structure specific recognition protein 1 (SSRP1) and contained a domain that was 47% identical to a portion of the HMG-domain protein HMG1.[273] Homologues of SSRP1 have been identified in both mouse and *Drosophila*.[274,275] The mouse homolog, T160, is involved in V(D)J recombination, but the function of the *Drosophila* homologue is unknown. A different type of screen involving damaged DNA-affinity precipitation techniques isolated two proteins of 26.5 and 28 kDa that bound to cisplatin-modified DNA, but not to DNA modified by *trans*-DDP.[276] These proteins were identified as HMG1 and HMG2 through amino-terminal sequence analysis. These initial experiments suggested that the family of HMG-domain proteins were strong candidates for binding preferentially to cisplatin-modified DNA and perhaps affecting its biological activities.

The HMG domain is a DNA−binding motif that consists of approximately 80 amino acids (for a review on HMG-domain proteins, see ref 277). A large superfamily of proteins contain this domain, including the prototypical members, the nonhistone chromosomal proteins, HMG1 and HMG2, as well as many transcription factors. Proteins in the family can contain multiple HMG domains and specifically recognize either DNA structures or sequences. They

bind in the minor groove and bend DNA upon binding. The ability of HMG-domain proteins to recognize distorted structures suggests why they may be able to bind to cisplatin-modified DNA (for reviews, see refs 278 and 279).

Many experiments were performed once the HMG-domain proteins were identified in the initial screening studies. Recombinant rat HMG1 bound specifically to cisplatin 1,2-intrastrand adducts, but not 1,3-intrastrand adduct or to *trans*-DDP modified DNA in gel mobility shift assays.[280] Studies using damaged DNA affinity precipitation also demonstrated that HMG1 and HMG2 bound preferentially to cisplatin-modified DNA.[281] The binding of HMG1 has been correlated with the length of the duplex DNA probe and also with the extent of cisplatin damage, a higher affinity of protein binding occurring with multiple cisplatin adducts.[282] HMG1 has also been claimed to bind cisplatin interstrand cross-links, although this result is puzzling in view of the structurally disparate nature of this and the 1,2-intrastrand cross-links.[270]

Screening of a yeast cDNA library in experiments similar to ones described for human cells led to the discovery of a yeast HMG-domain protein, Ixr1, that also bound preferentially to the cisplatin 1,2-intrastrand adducts.[283] Ixr1, also known as Ord1, is a transcription factor that binds to the Cox5b promoter and regulates transcription of an isoform of cytochrome *c* oxidase subunit V.[284] Gel mobility shift assays showed Ixr1 to bind at least an order of magnitude more tightly to cisplatin-modified compared to unmodified DNA.[285] The binding site of Ixr1 was revealed to be 15 bp in size centered around the platinum lesion through hydroxyl radical footprinting experiments.[285]

Aside from HMG1, HMG2, and Ixr1, many other HMG-domain proteins exhibit the same selectivity for cisplatin−DNA adducts. Some of these include HMG-D, a *Drosophila* homologue of HMG1;[286] mtTFA, a mitochondrial transcription factor;[287] hUBF, a ribosomal RNA transcription factor;[288] tsHMG, a testis-specific HMG protein;[289] SRY, the sex-determining factor;[290] and Cmb1, a yeast protein with mismatch-binding activity.[291] Perhaps most interesting on this list are tsHMG and SRY, owing to the sensitivity of testicular tumors to cisplatin. SRY is a human protein responsible for testis formation, and it has also been detected at the mRNA level in adult testis.[290] tsHMG is a mouse protein expressed in the nuclei of spermatocytes and elongating spermatids.[289] The sequence of this protein is nearly identical to that of mouse mtTFA. To date there is no human homolog. The binding affinity and specificity of tsHMG for cisplatin-modified DNA are much higher than for other HMG-domain proteins that have been studied.[289]

As mentioned above, HMG-domain proteins can contain multiple, usually tandem, HMG domains. The HMG domain is believed to be the DNA−binding element. Studies have been performed to look at individual domains binding to cisplatin-modified DNA. HMG1 contains two HMG domains, A and B, both of which bind preferentially to platinated DNA.[292,293] HMG1 domains A and B give the same DNase I footprint, both covering 15 nucleotides in the platinated strand and 12 residues in the complementary strand.[294] Hydroxyl radical footprinting with HMG1 domain A produces a different result with the protein protecting five bases on the platinated strand and four on the complementary strand.[295] This footprint is offset to the 3′-side of the *cis*-GG adduct, whereas the DNase I footprint is centered at the platination site. The discrepancies between these two experiments may reflect differences in the methods used. The size of the protected region in a DNase I footprint can be overestimated because the enzyme contacts two phosphate groups on each side of the hydrolyzed bond and two on the opposite strand across the minor groove.[296] In addition, DNase I prefers to cleave at the 5′-side of pyrimidines and between bonds with a high local twist angle.[297] More detailed structural studies, such as those discussed below, are required to resolve this issue.

Studies using HMG domains from tsHMG, SRY, and LEF-1 have demonstrated that these HMG domains can also bind specifically to cisplatin-modified DNA.[287,289,290] There is an important caveat in drawing conclusions from these studies, however. Recent work established that the sequence context surrounding the cisplatin adduct and the composition of the HMG domain itself can dramatically change the binding affinity for cisplatin-modified DNA.[293] For example, alteration of the nucleotides flanking a *cis*-GG adduct changed the binding affinity of HMG1 domain A by up to 2 orders of magnitude, and the binding of HMG1 domain A was 100-fold stronger than the binding of HMG1 domain B. In view of these results, much of the earlier work would have to be repeated with more careful control of the nature of the platinated DNA probe. The binding studies with the isolated HMG domains described above were performed by using gel mobility shift assays. The binding of these proteins has also been studied by using damaged DNA-affinity precipitation assays. Under these experimental conditions, the two individual domains of HMG2 bound weakly to cisplatin-modified DNA in the presence of 0.5 M NaCl, but a protein fragment containing both domains in tandem bound with high affinity.[298] Similar findings were reported with this assay for the HMG domains from HMG1, where the binding of the individual domains was abrogated by concentrations of NaCl greater than 0.3 M.[299]

These experiments demonstrate that, under some conditions, individual HMG domains bind cisplatin-modified DNA as well as the full length proteins. This result cleared the way for some meaningful structural work to be performed. Shorter peptide fragments are more amenable for use in such studies compared to full length protein, and any interactions observed with the isolated domains could be considered relevant to the binding of the full length protein. NMR solution structures of HMG1 domains A and B have been solved.[300−302] As indicated in Figure 19 for HMG1 domain B, both domains A and B have three α-helices forming the shape of an L with the angle between its arms being ∼80°.

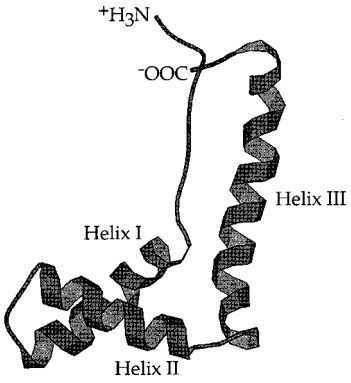


**Figure 19.** A MOLSCRIPT[329] representation of the NMR solution structure of HMG1 domain B.[301]

Later structural work showed the L-shaped α-helical fold to be common among HMG domains. Some of the first high-resolution structural studies of HMG-domain protein−DNA complexes were performed with the sequence-specific HMG-domain proteins. NMR solution structures were solved for the HMG domains of the proteins LEF-1 and SRY bound to their DNA target sequences.[303,304] In these structures, the L-shaped proteins bound in the minor groove, severely bending and unwinding the DNA. The domain from LEF-1 bent its DNA target by ∼117°, whereas the bend in the SRY DNA sequence was ∼70°−80°. In both cases, an amino acid side chain, methionine and isoleucine, respectively, intercalated into the DNA duplex from the minor groove side at the site of the bend. Some geometric information about the structure-specific HMG-domain protein−DNA complexes is also available. The NMR solution structure of the yeast HMG-domain protein NHP6A has been solved, and a model partially based on the NMR data of the protein−DNA complex has been constructed.[305] In this model, the protein binds in the minor groove, and the DNA is highly bent, as observed for the sequence-specific protein−DNA complexes. Methionine and phenylalanine side chains are proposed to intercalate into adjacent base pairs in the DNA. The positioning of the protein on the DNA differs from that of the sequence-specific SRY domain. In particular, it is shifted by 1 bp and binds in a reverse orientation. This model study provides some clues about the binding of structure-specific HMG-domain proteins to DNA.

The first structural information about the binding of a HMG domain to cisplatin-modified DNA was obtained through gel mobility shift studies (Table 4). The ability of several different full length HMG-domain proteins and some selected individual domains to bend cisplatin-modified DNA was investigated.[287] HMG1, mtTFA, and Ixr1 bent DNA by 86°, 87°−90°, and 68°, respectively. Slightly smaller bend angles were obtained with HMG domains from HMG1 (domain B), SRY, and LEF-1, where the cisplatin-modified DNA bent by 65°−74°, ∼50°, and 72°, respectively. The bend locus in all of these protein−DNA complexes was centered near the platinum adduct. Preliminary NMR work monitoring changes in chemical shifts suggested that HMG1 domain A bound to platinated DNA through the concave face of the protein.[306] Fluorescence resonance energy transfer (FRET) has also been employed to examine the bending that occurs when HMG1 domain B binds to a cisplatin-modified DNA probe.[307] These studies revealed the DNA to be bent by 80°−95° upon protein binding.

Although these preliminary studies afford some indication about the structure of complexes of platinated DNA with HMG-domain proteins, very recently an X-ray crystal structure was determined of HMG1 domain A bound to a 16-bp DNA probe containing a *cis*-GG adduct (Figure 20).[295] This structure shows the protein to bind through its concave surface and bend DNA by ∼61°. The protein bend is not centered at the platinum adduct, however, but is translocated by 2 bp to the 3′-side. This unique positioning also occurs in solution as confirmed by hydroxyl radical footprinting. An aromatic side chain, F37, intercalates into the DNA at a hydrophobic notch located in the minor groove across from the platinum adduct. Mutation of this side chain to an alanine residue substantially diminishes the binding affinity of this protein, suggesting that it is an important element for complex formation. The dihedral angle between guanine ring planes is 75°. This angle is larger than observed in the X-ray and NMR structures of the cisplatin-modified duplex DNA alone, making the geometry less constrained and similar to that encountered in the platinated dinucleotide d(pGpG) structure.[75,76] One of the ammine ligands is within hydrogen-bonding distance of a phosphate oxygen, as

**Table 4. Bend Angles for HMG-Domain Protein Complexes with Cisplatin-Modified DNA**

| protein | DNA size and flanking adduct sequence[a] | method | DNA bend angle (deg) | ref |
|---|---|---|---|---|
| HMG1 | 92-mer, AG*G*C | gel permutation | 86 | 287 |
| Ixr1 | 92-mer, AG*G*C | gel permutation | 68 | 287 |
| mtTFA | 92-mer, AG*G*C | gel permutation | 87−90 | 287 |
| HMG1 domain A | 16-mer, TG*G*A | X-ray | 61 | 295 |
| HMG1 domain B | 92-mer, AG*G*C | gel permutation | 65−74 | 287 |
| HMG1 domain B | 20-mer, TG*G*T | FRET | 80−95 | 307 |
| LEF-1 domain | 92-mer, AG*G*C | gel permutation | 72 | 287 |
| SRY domain | 92-mer, AG*G*C | gel permutation | 50 | 287 |
| LEF-1 domain[b] | 15-mer | NMR | 117 | 304 |
| SRY domain[b] | 8-mer | NMR | 70−80 | 303 |
| LEF-1 domain[b] | ∼100-mer | gel permutation | 102−125 | 287 |
| SRY domain[b] | ∼100-mer | gel permutation | 80 | 287 |

[a] The site of platination is denoted by asterisks. [b] These bend angles are for the HMG domains binding to their target DNA sequences or to a consensus sequence.

**Figure 20.** Structure of HMG1 domain A bound to d(CCTCTCTG*G*ACCTTCC)·d(GGAAGGTCCAGAGAGG) containing a *cis*-GG adduct, where G* denotes the location of platinated nucleotides.[295] The F37 residue, which intercalates into the platination site, is depicted in a space-filling representation. Reprinted with permission from ref 295. Copyright 1999 Macmillan Magazines Ltd.

was noted in earlier structures. This X-ray study provides the first detailed structural information for a complex between an HMG-domain protein and cisplatin-modified DNA and may be used in conjunction with other mechanistic work to help design more effective anticancer agents.

Thus far many studies have examined the binding affinity and structures of complexes between cisplatin-modified DNA and HMG-domain proteins. One area that is just now being explored is the kinetics of binding and dissociation for these protein−DNA complexes.[307] By using fluorescently labeled cisplatin-modified DNA probes in the FRET experiments described above, a fluorescence signal change was detected upon HMG-domain protein binding. Stopped-flow kinetic monitoring of the time dependence of this signal change afforded information about the rate at which an HMG-domain protein binds to and dissociates from cisplatin-modified DNA. With this methodology, the rate constant for HMG1 domain B binding to cisplatin-modified DNA was determined to be $1.1 \times 10^9$ $M^{-1}$ $s^{-1}$, and the rate constant for dissociation of the complex was 30 $s^{-1}$.[307]

Rate information is potentially important for understanding how HMG-domain proteins might participate in the cisplatin mechanism of action. Recent work has demonstrated that when RPA and HMG1 are both present, the latter selectively binds cisplatin-modified DNA at the expense of RPA complex formation.[260] One possible reason for this result is that HMG1 binding to cisplatin-modified DNA occurs at a faster rate than RPA binding.[260] The stopped-flow kinetic results for HMG1 domain B are consistent with this explanation since the associative rate constant value is near the diffusion limit.

### *2. Implications for the Mechanism of Action*

Several mechanisms have been considered for how HMG-domain proteins might modulate the sensitivity of cells to cisplatin. Two of the more prominent hypotheses have been explored through different experimental methods (see Figure 21). One hypothesis is that cisplatin−DNA adducts hijack proteins away from their normal binding sites, thereby disrupting cellular function. Since many HMG-domain proteins function as transcription factors, their removal from promoter or suppressor sequences by binding to cisplatin−DNA adducts could severely alter tumor cell biology. The other hypothesis, referred to as repair shielding, suggests that HMG-domain proteins could block cisplatin−DNA adducts from damage recognition needed for repair. This activity would result in diminished repair of the adducts, and persistence of platinum on the DNA could lead to cell death. It should be emphasized that these two models are not mutually exclusive and could work in concert to effect cisplatin cytotoxicity.

For the hijacking hypothesis, the binding affinity of an HMG-domain protein for cisplatin-modified DNA, adjusted for the relative number of binding sites, would have to be comparable to that of the natural binding site of the protein. Early work with the human upstream binding factor (hUBF), involved in ribosomal RNA transcription, demonstrated that this particular HMG-domain protein has a high affinity for cisplatin-modified DNA,[288] comparable to its affinity for the promoter. Later work showed that this enhanced binding affinity might be due to the presence of multiple HMG domains in the protein.[138] In an in vitro ribosomal RNA transcription assay, cisplatin, but not *trans*-DDP, inhibited the synthesis of rRNA in a dose dependent manner.[138] This reconstituted system requires the presence of hUBF, RNA polymerase I, and SL1, which is composed of the TATA binding protein (TBP) and associated factors. Addition of excess hUBF to the cisplatin-inhibited reactions restored the rRNA transcription, suggesting that hUBF had been hijacked away from its normal binding site to the cisplatin−DNA adducts. One interesting note about this study is that, in addition to hUBF, TBP binds preferentially to cisplatin-modified DNA, as will be discussed below.[308] The fact that addition of hUBF alone restored transcriptional activity implies that hijacking of TBP from its normal binding site is not an important component in this transcriptional inhibition by cisplatin.

More recently, treatment of cells with cisplatin blocked the synthesis of rRNA in vivo and caused a redistribution of hUBF and the other components of rRNA transcription in the nucleolus.[309] As with the in vitro assay, these effects were not observed with *trans*-DDP. This result could be consistent with a hijacking mechanism, although the experiments indicated that hUBF is not removed from the nucleolus to other sites in the nucleus. Alternatively, as preferred by the authors, the presence of hUBF could shield these cisplatin adducts from repair, causing

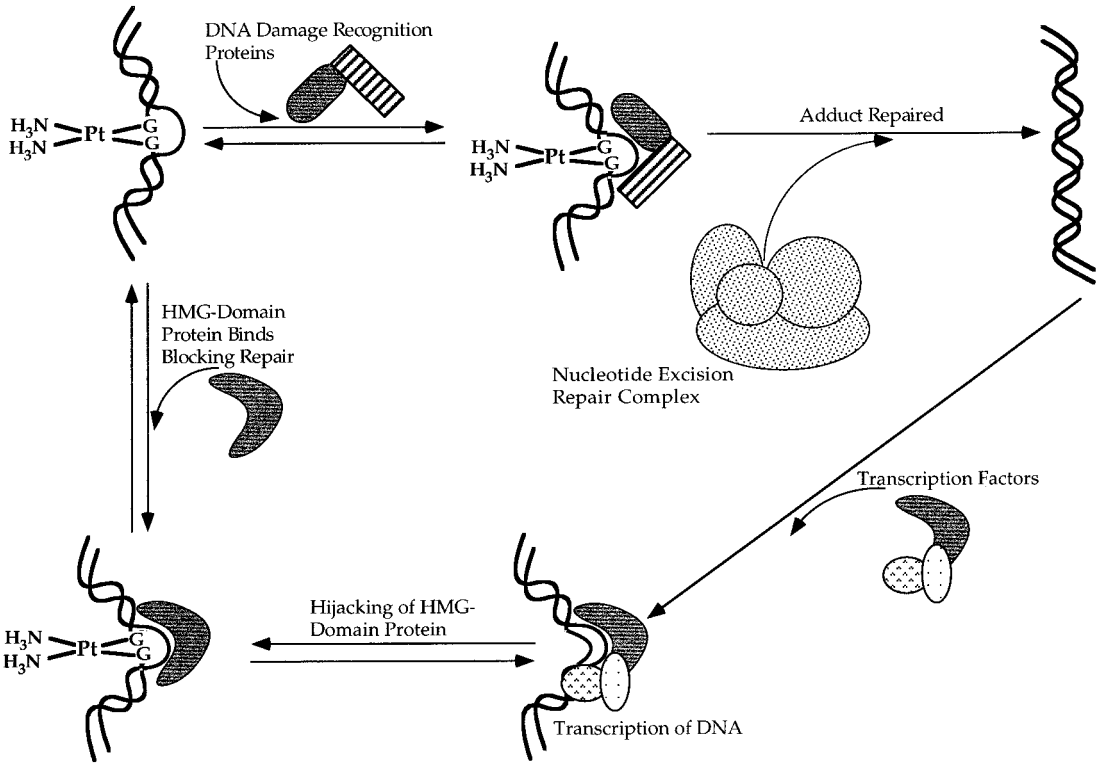


**Figure 21.** A schematic diagram of the hijacking and repair shielding hypotheses proposed for how HMG-domain proteins might mediate cisplatin cytotoxicity.

the lesion to persist in the nucleolus while DNA in other regions of the nucleus are more effectively repaired.[309]

Experimental evidence has also been obtained for the repair shielding hypothesis. Yeast mutants lacking the Ixr1 protein were 2−6-fold less sensitive to cisplatin than wild-type cells.[283,310] These results were not observed with *trans*-DDP and suggested that Ixr1 might shield cisplatin adducts from repair, sensitizing the cells to the drug. This suggestion was later linked to the nucleotide excision repair pathway, because the differential sensitivity was not observed in yeast cells deficient in components involved in NER steps up to the first irreversible one, the 3′-incision by Rad2.[310] This system was also employed to investigate the hijacking hypothesis. Experiments demonstrated that treatment of cells with cisplatin did not cause Ixr1, which is involved in Cox5b transcription, to be diverted from its normal binding site sufficiently to disrupt Cox5b mRNA formation from genomic DNA or from a reporter gene under the control of the Cox5b promoter.[285] Finally, more direct in vitro experiments showed that by adding HMG-domain proteins, such as HMG1, mtTFA, tsHMG, HMG1 domain B, and SRY, to the nucleotide excision repair assay, removal of *cis*-GG adducts could be blocked in a dose dependent fashion.[170,171,176,290] Also in agreement with the repair shielding model is the finding that the immunoprecipitation of HMG1 and HMG2 from cell extracts enhances repair synthesis activity for cisplatin-modified DNA.[311]

The experiments presented above provide some evidence for how HMG-domain proteins might play a role in the cisplatin mechanism. As the functions of structure-specific HMG-domain proteins are uncovered, new insight into how these proteins might affect cisplatin activity may be afforded. For example, SSRP1, the first HMG-domain protein found to bind selectively to cisplatin−DNA adducts, has recently been demonstrated to be one of a two-component factor required for transcription elongation from chromatin, suggesting a specific pathway that might be affected by this drug.[312] Although the hypothesis that these proteins are important mediators of cisplatin cytotoxicity remains to be proved, a large body of experimental evidence points to their likely importance. The intracellular distribution of HMG1, HMG2, and hUBF changes following treatment of cells with cisplatin.[309,313] Overexpression of tsHMG in HeLa cells modulates the cytotoxic properties of the drug,[314] and the cisplatin sensitivity of non-small cell lung cancer cells was increased more than 3-fold by the introduction of the HMG2 gene.[315] Examination of the intracellular platinum concentration and glutathione levels in the latter study indicates that these two factors cannot account for the increase in cisplatin sensitivity, consistent with the idea that HMG-domain proteins are shielding cisplatin−DNA adducts from repair. HMG1 mRNA levels in the human breast cancer cell line, MCF-7, are increased 2.5-fold after 0.5 h of estrogen treatment and remain 1.5-fold higher than basal levels from 1.5 to 24 h with continuous estrogen treatment.[316] Cotreatment of MCF-7 cells with cisplatin and estrogen causes a 2-fold increase in drug sensitivity.[317] Treatment of MCF-7 cells with estrogen and *trans*-DDP did not produce a sensitivity change, and the cisplatin sensitivity of HeLa Cells, which do not have an estrogen receptor, is not affected by estrogen.[317]

The work discussed above indicates that HMG-domain proteins could be important in modulating the toxicity of cisplatin. There are, however, studies that are not consistent with this hypothesis. Repair assays performed with cell extracts fractionated to exclude potential shielding proteins did not change the relative repair levels of the 1,2- and 1,3-intrastrand cisplatin adducts.[172] The relative repair rates of these adducts in a reconstituted system of highly purified components was similar to that in whole cell extracts.[171] In both of these studies, the differential repair was attributed to the different DNA structures induced by the cisplatin adducts rather than to the shielding of selected adducts by cellular proteins. Thus, while not supporting the repair shielding hypothesis, these results do not directly contradict it since the repair of two different adducts are being compared. Experiments performed in yeast cells lacking the HMG-domain protein Cmb1 revealed that the mutant cells were more sensitive to cisplatin than the wild-type cells expressing the HMG-domain protein, whereas no difference was seen between the two cell lines with *trans*-DDP.[291] These results are the opposite of what was seen when yeast mutants lacking the Ixr1 protein were treated with cisplatin and suggest that Cmb1 could play a role in facilitating repair of the platinum lesions rather than shielding the adducts from repair.

Although the role of HMG-domain proteins in the anticancer mechanism needs to be explored further, there is reason to believe that this class of proteins could be engineered to modulate the response of cells to cisplatin. A treatment strategy involving a combination of gene therapy and chemotherapy, where HMG-domain protein levels are raised in conjunction with cisplatin administration, has the potential to sensitize cells to the drug. Either the domain or the platinum complex could be altered to improve the specificity of binding in vivo with the goal of improving the therapeutic response.

### C. Transcription Factors Lacking an HMG Domain

Aside from repair and HMG-domain proteins, other cellular factors bind preferentially to cisplatin-modified DNA. The TATA binding protein (TBP) is one of these. TBP is part of the basal transcription factor TFIID. Both TFIID and TBP bind selectively to DNA damaged by cisplatin and UV radiation.[308] The affinity of this protein for the 1,2-intrastrand adducts of cisplatin is greater than for the 1,3-intrastrand adduct or for DNA modified by *trans*-DDP, just as was observed with the HMG-domain proteins.[318] It has been suggested that TBP recognizes distorted DNA structures that are similar to the complex it makes upon binding to the TATA box.[318] The preference of TBP for bent DNA has been observed previously.[319,320] Binding to damaged DNA has the potential to titrate this transcription factor away from its normal binding site and interfere with transcription. In support of this theory, microinjection of TBP into cells treated with UV radiation reduced the inhibition of RNA synthesis.[308] Another possible option is that TBP could block the recognition of cisplatin adducts from the excision repair proteins. Further studies are required before the potential role of TBP in the cisplatin mechanism can be assessed.

Another transcription factor that has recently been shown to recognize cisplatin-modified DNA is the Y-box binding protein (YB-1).[321] YB-1 is a transcription factor that binds to the Y-box, also known as the inverted CCAAT box, found in the promoter regions of many eukaryotic genes. This protein also binds specifically to single-stranded and apurinic DNA.[322] The preference for cisplatin-modified DNA over unmodified DNA was demonstrated in gel mobility shift assays, but there was no specificity for binding the *cis*-GG and *cis*-AG adducts over the *cis*-GNG adduct, as was seen with the HMG-domain proteins.[321] YB-1 is overexpressed in some cisplatin-resistant cell lines, and the sensitivity of these cells could be increased by using a YB-1 antisense construct.[323] This protein also interacts with PCNA, a component necessary for NER.[321] Thus, this initial work suggests that YB-1 could be part of the cisplatin mechanism, although more studies are needed to establish its potential role.

### D. Architectural Proteins without an HMG Domain

In addition to TBP, the histone H1 binds to cisplatin-damaged DNA.[324,325] This histone interacts with linker DNA connecting nucleosome core particles in the chromatin fiber and has binding properties similar to that of HMG1 and HMG2.[324] In a competition experiment, histone H1 bound to cisplatin-modified DNA more strongly than HMG1.[324] The specificity of this protein for individual cisplatin−DNA adducts has yet to be determined, however. It will be interesting to see whether this protein has the same specificity as the HMG-domain proteins and TBP. Histone H1 (approximately one per nucleosome)[326] is a more abundant chromatin protein than HMG1 and HMG2 (about one per 20 nucleosomes),[327] although little is known about the availability of the free nuclear proteins.[327] The ~10-fold greater nuclear abundance of histone H1 compared to HMG1 and HMG2 makes it a tempting prospect for participating in the cisplatin mechanism.[253] The precise role these proteins might play in the cytotoxicity of cisplatin is still unknown, but it is conceivable that some of the same mechanisms suggested for the HMG-domain proteins could apply for these proteins as well.

## *VI. Conclusions and Future Directions*

Over the past ~35 years since the discovery of the biological activity of cisplatin, much has been learned about how this compound affects cells. DNA has been identified as a key cellular target of this drug, and many detailed structural studies have provided important information about DNA distortions induced by cisplatin lesions. The effects of cisplatin on DNA function have been studied, and there is also some information about how cisplatin−DNA adducts are processed in cells. The past 10 years have witnessed a successful search for cellular proteins that bind specifically to cisplatin−DNA adducts. Several such proteins have been identified to date, and studies

have been performed to determine how they might facilitate the cytotoxic properties of the drug.

Despite the information that has been acquired thus far, there are still many aspects of the cisplatin mechanism that remain a mystery. Pathways of cisplatin resistance, both intrinsic and acquired, are not well-understood at the molecular level, due to the many factors that could contribute to this condition. Alternative cellular DNA targets, such as telomeres and mtDNA, have only recently been investigated and may prove to be important. Proteins that bind to cisplatin-modified DNA continue to be identified and have the potential to play important roles in the mechanism of action. These kinds of cellular interactions need to be explored in more detail in order to understand how cisplatin kills cells.

The information gathered to date about the cisplatin mechanism has the potential to improve platinum antitumor drug therapy. Since overexpression of an HMG-domain protein in cells modulates cisplatin sensitivity, this concept could in principle be extended to a combination gene therapy−chemotherapy protocol. The design of new mechanistic based screening methods, like those described above with the green fluorescent protein and $\beta$-lactamase, may identify new platinum drug candidates from combinatorial libraries having an expanded organotropic profile. Continued research into the mechanism of action of cisplatin may allow us to understand why this compound is so extraordinarily effective against testicular cancer. With this knowledge, it should be possible to design rationally new drugs that treat many types of tumors with reduced toxic side effects and to overcome the resistance problems encountered with the current platinum anticancer drugs.

## VII. Acknowledgments

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