The Role of Base Excision Repair Proteins in the Cellular Responses

to the Anticancer Drug Cisplatin

by

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Abstract

Cisplatin is one of the most widely used anticancer drugs. Its success in killing tumor cells results from its ability to form DNA adducts and the cellular processes triggered by the presence of those adducts in DNA. The geometric isomer of cisplatin, *trans*-DDP can also form DNA adducts but fails to show any antitumor activity. Variations in tumor response may result from altered expression of cellular proteins that recognize cisplatin and *trans*-DDP adducts.

The human 3-methyladenine DNA glycosylase (AAG) is a repair enzyme that removes a number of damaged bases from DNA including adducts formed by some chemotherapeutic agents. Using site-specifically modified oligonucleotides containing each of the cisplatin intrastrand crosslinks we found that AAG readily recognized cisplatin adducts. The apparent dissociation constants for the 1,2-d(GpG), the 1,2-d(ApG) and the 1,3-d(GpTpG) oligonucleotides were 115 nM, 71 nM, and 144 nM, respectively. For comparison, the apparent dissociation constant for an oligonucleotide containing a single $1,N^6$-ethenoadenine (εA), which is repaired efficiently by AAG, was 26 nM. Despite the affinity of AAG for cisplatin adducts, AAG was not able to release any of these adducts from DNA. Furthermore, it was demonstrated that the presence of cisplatin adducts in the reactions inhibited the excision of εA by AAG. These data suggest a previously unexplored dimension to the toxicological response of cells to cisplatin. We suggest that cisplatin adducts could titrate AAG away from its natural substrates resulting in higher mutagenesis and/or cell death

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because of the persistence of AAG substrates in DNA.

The *mutM* and *mutY* genes encode DNA glycosylases involved in base excision repair; these genes constitute part of the "GO" repair system that, heretofore, has been associated with cellular defenses against the oxidative lesion, 7,8-dihydro-8-oxoguanine (8-oxoG). The MutM protein removes ring-opened purines and 8-oxoG from DNA. The MutY protein removes adenine from A:G and A: 8-oxoG mismatches. Purified MutM protein recognized the intrastrand crosslinks formed by cisplatin but had no excision activity on these adducts. Furthermore, *in vivo* experiments with wild type and *mutM* cells demonstrated that the presence of the MutM protein sensitized cells to cisplatin, and this effect of MutM was essentially abolished in a nucleotide excision repair deficient background, suggesting that MutM somehow modulates the activity of nucleotide excision repair proteins on cisplatin adducts. Purified MutY was also able to recognize cisplatin adducts. Moreover, MutY could excise adenine incorporated opposite a platinated guanine and was able to excise the platinated adenine of the 1,2-d(ApG) adduct when paired with guanine, suggesting that MutY might be directly involved in the repair of cisplatin adducts *in vivo*. These interactions between MutY and cisplatin adducts appears to be very important since *mutY* cells were significantly more sensitive to cisplatin than wild type cells. Moreover, expression of MutY or p26, but not D138N p26, in *mutY* cells rescued their sensitivity to cisplatin, indicating that the catalytic activity of MutY is important for survival after treatment with cisplatin. Interestingly, MutY was also required for cisplatin induced mutagenesis, whereas MutM did not appear to play a role. Finally, a *mutM* or *mutY* mutation did not affect survival to *trans*-DDP, and MutM and MutY proteins displayed higher affinity for cisplatin than *trans*-DDP adducts.

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This thesis is dedicated to my parents.

All four of them...

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List of Abbreviations

1,2-d(ApG)	*cis*-[Pt(NH$_3$)$_2${d(ApG)-N7(1),-N7(2)}]
1,2-d(GpG)	*cis*-[Pt(NH$_3$)$_2${d(GpG)-N7(1),-N7(2)}]
1,3-d(GpTpG)	*cis*-[Pt(NH$_3$)$_2${d(GpTpG)-N7(1),-N7(3)}]
3-mA	3-methyladenine
3-mG	3-methylguanine
5^{OH}C	5-hydroxycytosine
5^{OH}U	5-hydroxyuracil
6-TG	6-thioguanine
7-mG	7-methylguanine
8-oxoA	7,8-dihydro-8-oxoadenine
8-oxoG	7,8-dihydro-8-oxoguanine
AAG	human 3-methyladenine DNA glycosylase
Aag	mouse 3-methyladenine DNA glycosylase
AP	apurinic/apyrimidinic
BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
cisplatin or *cis*-DDP	*cis*-diamminedichloroplatinum(II)
DACH or Pt(dach)Cl$_2$	*cis*-diaminocyclohexanedichloroplatinum(II)
dien or [Pt(dien)Cl]$^+$	chlorodiethylenetriamineplatinum(II)
DMS	dimethyl sulfate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
εA	1,N^6-ethenoadenine
EDTA	ethylenediamine tetraacetic acid

EGTA	ethylene glycol-bis(2-aminoethyl)- N, N, N',N'-tetraacetic acid
en or Pt(en)Cl$_2$	ethylenediaminedichloroplatinum(II)
fpg	formamidopyrimidine DNA glycosylase
HMG	high mobility group
Hx	hypoxanthine
ICL	interstrand crosslink
IPTG	isopropylthio-β-D-galactoside
Ixr1	intrastrand crosslink recognition protein
Kd	dissociation constant
LB	Luria Bertami broth
LEF-1	lymphoid enhancer binding factor 1
MGMT	0^6-methylguanine methyltransferase
MMC	mitomycin C
MMS	methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
mRNA	messenger RNA
mtTFA	mitochondrial transcription factor
MUG glycosylase	G:T/U mismatch specific DNA glycosylase
N-SCLC	non-small cell lung cancer
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol, average M.W. 8,000
RNA	ribonucleic acid
SCLC	small cell lung cancer
SSRP1	structure specific recognition protein 1
TAE	200 mM Tris, 0.57% glacial acetic acid, 0.2 mM EDTA

TBE	89 mM Tris, 89 mM boric acid, 0.2 mM EDTA
TE	10 mM Tris-Cl, 1 mM EDTA
trans-DDP	*trans*-diamminedichloroplatinum(II)
Tris-Cl	tris(hydroxymethyl)aminomethane hydrochloride
UBF	upstream binding factor
XP	Xeroderma Pigmentosum

Chapter 1

Literature Survey

A. Introduction

The biological activity of *cis*-diamminedichloroplatinum(II) or cisplatin was discovered serendipitously in 1965 by Barnett Rosenberg during studies on the effects of electric current on bacterial growth. Rosenberg noticed that the application an of electric field caused *Escherichia coli* cells to form long filaments up to 300 times the length of a normal cell (Rosenberg et al., 1965). Further investigation indicated that a platinum salt produced at the electrode during electrolysis was the active agent responsible for this phenomenon (Rosenberg et al., 1967). A number of different platinum complexes were tested for biological activity, and several of them, including cisplatin (Howle and Gale, 1970), were able to induce filamentous growth in bacteria. Interestingly, the *trans* isomer of cisplatin, *trans*-diamminedichloroplatinum(II) did not induce filamentous growth but merely acted as a bactericide. Subsequent studies have revealed that cisplatin inhibits DNA replication, induces prophage from lysogenic *E. coli* (Reslova, 1971) and, most importantly, has antitumor activity. Cisplatin inhibits the growth of Sarcoma 180 cells and leukemia L1210 cells in mice (Rosenberg et al., 1969). Moreover, cisplatin is effective against the Dunning ascitic leukemia (Kociba et al., 1970), the Walker 256 carcinosarcoma (Kociba et al., 1970), and a methylbenzanthracene induced mammary carcinoma (Welsch, 1971) in rats. Clinical studies commenced soon after the demonstration of activity against a broad spectrum of animal tumors and in 1978 cisplatin was approved by the FDA. Today cisplatin is one of the most widely used anticancer agents. For a historic review of the discover of cisplatin refer to (Rosenberg, 1985).

Cisplatin is particularly effective in the treatment of testicular tumors where it can afford cure

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rates greater than 95% (Feuer et al., 1993). It is noteworthy that prior to the introduction of cisplatin for the treatment of testicular cancer, the survival of patients with advanced non-seminomatous testicular cancer was very low, and few patients were expected to survive after 2 years. Today, according to estimates from the National Cancer Institute, the 5 year survival rate of patients with Stage III non-seminomas after treatment with cisplatin based chemotherapy has increased to 70%. Additionally, cisplatin is routinely used in the treatment of ovarian, bladder, head and neck, lung cancer, and there are numerous clinical trials underway to investigate the efficacy of cisplatin in the treatment of other cancers and to evaluate potential modulators of cisplatin antitumor activity.

Because of some resemblance to nitrogen mustards, which form intrastrand and interstrand crosslinks, it was suggested quite early on that the target of cisplatin was DNA. Studies with *E. coli* and eukaryotic cells demonstrated that DNA repair deficient cells are more sensitive to cisplatin than wild type cells indicating that DNA is indeed the principal target of cisplatin. Moreover, there is a correlation between DNA adduct levels and survival after cisplatin treatment. Finally, at pharmacologically relevant doses of cisplatin, the levels of binding to RNA and protein are too low to inactivate such molecules. Consequently, a number of studies has focused on elucidating the interactions of cisplatin with DNA. One approach involves studying the structural features of cisplatin DNA adducts and determining how they are processed by cellular proteins.

B. Adducts Formed by Cisplatin and *trans*-DDP

Cisplatin is a neutral, square planar coordination complex of platinum (II). It is coordinated to two chloride and two ammonia groups in the cis geometry. The ammonia groups are strongly

bound to platinum(II), whereas the chloride ligands are easily substituted by nucleophiles. In aqueous solution both chloride ions slowly dissociate from the coordination sphere of platinum(II) and get replaced by water or hydroxide ions, resulting in an equilibrium of unhydrolyzed, partially hydrolyzed, or fully hydrolyzed platinum species. The equilibrium composition is very sensitive to the concentration of chloride ions, and at high chloride concentrations the majority of the species in solution is unhydrolyzed cisplatin.

Cisplatin is administered intravenously and, while it circulates in the blood, where the chloride concentration is high (~100 mM), Pt(II) remains coordinated to its chloride ligands. However, upon entering the cell where the chloride concentration is low (4 mM), the chloride ligands get replaced by water molecules ($t_{1/2}$ = 2 hrs for substitution of the first chloride ligand with water). It is this positively charged aquated species that can react with nucleophilic sites on macromolecules to form protein, RNA and DNA adducts. The water molecule of the mono-aquated species is far more reactive than the chloride ligand, and it is easily replaced by the N7 of guanine or adenine forming a monofunctional adduct. Then, if there is another nucleophilic site nearby, the monofunctional adduct reacts further to form intrastrand and interstrand crosslinks.

The relative amount of adducts formed by cisplatin was determined by treating salmon sperm DNA in vitro, enzymatically digesting the DNA to nucleotides, separating the products by FPLC, and characterizing the adducts by their ¹H NMR spectra. It was thereby shown that cisplatin treated DNA contains approximately 65% 1,2-d(GpG), 25% 1,2-d(ApG), and 5-10% 1,3-d(GpNpG) intrastrand crosslinks, and a small percentage of interstrand crosslinks and monofunctional adducts

(Bruhn et al., 1990; Eastman, 1983; Eastman, 1985; Fichtinger-Schepman et al., 1987; Fichtinger-Schepman et al., 1985; Fichtinger-Schepman et al., 1990; Plooy et al., 1985a; Zou et al., 1994). Significantly, when studies were performed using DNA isolated from leukocytes of patients treated with cisplatin, similar adduct profiles were observed.

The adduct spectrum of *trans*-DDP is not as well characterized. *trans*-DDP interacts with the N7 of purines or the N3 of cytosine, and it primarily forms intrastrand crosslinks between two guanine residues or a guanine and a cytosine separated by at least one base, and interstrand crosslinks between complementary guanine and cytosine residues (Eastman and Barry, 1987; Eastman et al., 1988; Brabec and Leng, 1993). Enzymatic digestion of double stranded DNA treated with *trans*-DDP *in vitro*, yields approximately 50% *trans*-[Pt(NH$_3$)$_2$\{d(GMP)\}\{d(CMP)\}], 40% *trans*-Pt(NH$_3$)$_2$\{d(GMP)$_2$\}\{d(CMP)\}], and 10% *trans*-[Pt(NH$_3$)$_2$\{d(GMP)\}\{d(AMP)\}] (Eastman et al., 1988). Recent quantitation studies revealed that interstrand crosslinks comprise 20% of *trans*-DDP adducts (Brabec and Leng, 1993).

C. Structure of Cisplatin and *trans*-DDP Adducts

The structural alterations induced in DNA after binding of platinum have been extensively studied. Gel electrophoresis studies revealed that all the cisplatin adducts bend and unwind the DNA helix. Rice *et al.* (Rice et al., 1988) studied the electrophoretic mobility of multimers of a 22 base pair (bp) oligonucleotide that contained a single 1,2-d(GpG) adduct, and analysis of their results led to the conclusion that the 1,2-d(GpG) cisplatin adduct bends the DNA helix by 40° towards the major groove. The 1,3-d(GpTpG) adduct induces a bend angle of 25-35° (Bellon and Lippard, 1990; Anin

and Leng, 1990). Schwartz *et al.* (Schwartz et al., 1989) compared the electrophoretic mobility of multimers of an oligonucleotide containing a single 1,2-d(GpG) or a single 1,2-d(ApG) adduct and showed that the two adducts induce the same distortion in the DNA helix, and a subsequent investigation demonstrated that these adducts bend the DNA helix by 32-34° (Bellon and Lippard, 1990). The interstrand crosslink of cisplatin bends the DNA helix by 45° (Malinge et al., 1994). Cisplatin adducts also unwind the DNA helix. The 1,2-d(GpG) and the 1,2-d(ApG) adducts unwind the DNA helix by 13°, the 1,3(GpTpG) adducts unwinds the DNA by 23° (Bellon et al., 1991), whereas the interstrand crosslink unwinds DNA by 79° (Malinge et al., 1994).

The adducts of *trans*-DDP also induce structural alterations in DNA. The 1,3-adduct of *trans*-DDP bends the DNA helix, but the bending does not have direction; i.e., the adduct serves as a hinge joint and increases DNA flexibility. The interstrand crosslink of *trans*-DDP bends the DNA helix towards the major groove by 26° and unwinds it by 12° (Brabec and Leng, 1993).

The X-ray structure of a double stranded dodecamer DNA containing a single, centrally located 1,2-d(GpG) cisplatin adduct has been solved (Takahara et al., 1995; Takahara et al., 1996). The adduct causes a 50° bend towards the major groove, thereby compressing the major groove and causing a widening and flattening of the minor groove. The dihedral angle between the guanine bases is 30°. The NMR solution structure of the same dodecamer reveals an overall helix bend of 78° and a 25° unwinding of the helix at the site of platination (Gelasco and Lippard, 1998). The dihedral angle between the guanine bases is 47° (Gelasco and Lippard, 1998). The base pairing at the site of platination is more distorted than in the X-ray structure (Takahara et al., 1995; Takahara

et al., 1996; Gelasco and Lippard, 1998). The NMR solution structure of an octamer duplex DNA has also been determined (Yang et al., 1995). The DNA is bent by 58° towards the major groove and unwound by 21°, causing a widening of the minor groove. The dihedral angle between the guanine bases is 59°.

Because of the structural similarities observed between 1,2-d(GpG) and 1,2-d(ApG) adducts in bending and unwinding studies, most research has focused on the most abundant 1,2-d(GpG) adduct, and, therefore, there is no high resolution structure of the 1,2-d(ApG) adduct. Molecular modeling studies based on the NMR spectrum of an oligonucleotide containing a single 1,2-d(ApG) adduct indicate that the DNA is bent by 55° towards the major groove, suggesting that the two 1,2-intrastrand crosslinks induce similar alterations in the DNA architecture.

Two NMR solution structures of duplex DNA containing a single 1,3-d(GpTpG) cisplatin adduct have been reported (van Garderen and van Houte, 1994; Teuben et al., 1999). The helix is unwound locally at the platination site and is bent towards the major groove, and the minor groove is widened. The overall structure is more distorted than the structure of DNA containing a single 1,2-d(GpG) adduct. The base pairing is lost at the 5' platinated guanine as well as in the central thymine: adenine base pair, and the central thymine is extruded in the minor groove.

The NMR solution structure of duplex DNA containing a single interstrand crosslink of cisplatin has also been reported (Huang et al., 1995; Paquet et al., 1996). The cis-diammineplatinum(II) bridge lies in the minor rather than the major groove, and the helix is bent (20°

or 40°) towards the minor groove. A net unwinding of 76° or 87° is observed. The complementary cytosines are extrahelical. Similar structural alterations are observed in the crystal structure of duplex DNA containing an interstrand crosslink (Coste et al., 1999). The DNA helix is bent by 47° towards the minor groove and is unwound by 70°, and the minor groove is enlarged. The complementary cytosines are extruded from the double helix and exposed to solvent.

The structure of a DNA duplex containing a single interstrand crosslink of *trans*-DDP has been studied by two-dimensional NMR (Paquet et al., 1999). The duplex is distorted over two base pairs on either side of the adduct, and it is bent by 20° towards the minor groove. The platinated guanine adapts a syn conformation. The rotation, mediated by the platinum moiety, results in a Hoogsteen-type pairing between the complementary platinated guanine and platinated cytosine.

It is obvious from the aforementioned studies that each cisplatin adduct distorts the DNA architecture in a unique manner; therefore, it is reasonable to expect that these adducts would be differentially recognized and processed by cellular proteins.

D. DNA Repair Mechanisms

All organisms have evolved DNA repair mechanisms with components that have been conserved through evolution to protect the integrity of their genome. To date two major excision repair pathways for damaged DNA have been identified. The first is nucleotide excision repair, which is usually involved in the repair of adducts that distort the DNA architecture and block DNA replication. The second pathway is base excision repair, which is usually involved in the repair of

bases that do not cause major helix distortions and frequently arise in DNA as a result of deamination, oxidation or alkylation processes that occur in cells. Other repair pathways include mismatch repair and recombination (Friedberg et al., 1995; Lindahl and Wood, 1999).

Nucleotide Excision Repair

Nucleotide excision repair involves the hydrolysis of two phosphodiester bonds, one on either side of the damaged DNA, resulting in the excision of an oligonucleotide containing the lesion. The excised oligonucleotide is then released from the duplex, and the resulting gap is filled in and ligated to complete the repair reaction. Three proteins in *E. coli*, UvrA, UvrB, and UvrC, are necessary and sufficient for the excision of the oligonucleotide (Sancar and Rupp, 1983; Yeung et al., 1983). These proteins are collectively called the Uvr(A)BC excinuclease. UvrA is a DNA dependent ATPase that serves in damage recognition. It forms a $(UvrA)_2$ UvrB complex that binds to unmodified DNA with relatively high affinity (Oh and Grossman, 1987). Upon encountering a lesion, the complex causes unwinding of 5 bp around the lesion and kinking of the DNA by 130° towards the major groove (Lin et al., 1992; Shi et al., 1992; Visse et al., 1994). Moreover, a conformational change in UvrB takes place that enables it to form a tight complex at the lesion site. After dissociation of UvrA (Orren and Sancar, 1989; Orren et al., 1992; Visse et al., 1992) and association of UvrC (Bertrand-Burggraf et al., 1991; Hsu et al., 1995), UvrB makes the 3' incision that causes a conformational change in UvrC enabling it to make the 5' incision (Lin and Sancar, 1990; Lin et al., 1992). UvrD releases the excised oligomer and promotes UvrC dissociation. Polymerase I displaces UvrB and fills in the gap, and a DNA ligase completes the repair reaction.

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Nucleotide excision repair is believed to be the main process by which platinum adducts are removed from DNA. Bacterial *uvrA*, *uvrB* or *uvrC* cells are more sensitive to cisplatin than wild type cells (Beck and Brubaker, 1973; Konishi et al., 1981; Alazard et al., 1982; Beck et al., 1985; Fram et al., 1986; Popoff et al., 1987). The *uvrA* cells show modest sensitivity to *trans*-DDP (Alazard et al., 1982; Fram et al., 1986). Moreover, when plasmid DNA is damaged with cisplatin or *trans*-DDP and is used to transform wild type, *uvrA,* or *uvrB* cells, the transformation efficiency, which serves as an indicator of the ability of a cell to repair damage, is higher in wild type cells, implying that nucleotide excision repair proteins can remove cisplatin and *trans*-DDP adducts (Husain et al., 1985; Popoff et al., 1987). In addition, biochemical experiments have demonstrated that plasmid DNA modified with cisplatin or *trans*-DDP is a substrate for the Uvr(A)BC excinuclease *in vitro* (Husain et al., 1985; Beck et al., 1985). Finally, the ability of Uvr(A)BC to repair a single 1,2-d(GpG) or a single 1,3-d(GpCpG) adduct was determined. The 1,2-d(GpG) adduct was a substrate for Uvr(A)BC (Visse et al., 1991){Visse, de Ruijter, et al. 1992 ID: 1523}and it was incised 3.5 fold more efficiently than the 1,3-d(GpCpG) adduct, and this difference in incision efficiency could not be attributed to differences in the rate of UvrC binding to the preincision complex (Visse et al., 1994).

The mammalian nucleotide excision repair system is far more complicated and is thought to require the coordinated action of at least 30 polypeptides (Mu et al., 1995; Aboussekhra et al., 1995). Defective excision repair in humans is associated with several diseases, including Xeroderma Pigmentosum (XP). Human excision repair genes include those defined by the seven complementation group of XP (A through G) and the excision repair cross complementing (ERCC)

genes. For a detailed review of nucleotide excision repair refer to (Wood, 1996; Sancar, 1996). Briefly, recognition of the damage is made by a complex of the XPA protein with the three subunits of replication protein A (RPA). The TFIIH protein complex and the XPE protein may also participate in damage recognition. Following recognition, incision steps at two sites flanking the lesion are made by XPG and the ERCC1-XPF complex. The completion of the repair reaction involves participation of the XPB and XPD helicases, proliferating cell nuclear antigen (PCNA), RPA, replication factor C, polymerase δ or ϵ, and one of the DNA ligases.

Mammalian cells deficient in nucleotide excision repair are more sensitive to cisplatin than the corresponding wild type cells. Initial studies compared the survival of human fetal lung cells and human skin fibroblast cells of complementation group A (XPA$^{-/-}$). When survival was expressed as a function of the amount of platinum bound to DNA, the XPA$^{-/-}$ cells were 4 fold more sensitive to cisplatin (Fraval et al., 1978). Further studies used normal and Xeroderma Pigmentosum fibroblasts to determine survival after cisplatin treatment. In these experiments XPA cells were 3 to 6 fold more sensitive than normal wild type cells (Poll et al., 1984; Dijt et al., 1988). It is noteworthy that to date the survival of isogenic XPA$^{+/+}$ and XPA$^{-/-}$ cells after treatment with cisplatin has not been compared. Moreover, Chinese hamster ovary cells deficient in ERCC1 protein (ERCC1$^{-/-}$ cells) transformed with human ERCC1 are 5 fold more resistant to cisplatin than ERCC1$^{-/-}$ cells (Lee et al., 1993).

In vitro repair assays confirmed that cisplatin adducts are substrates for the human nucleotide excision repair machinery. Extracts from wild type cells elicit repair synthesis when incubated with plasmid DNA modified with cisplatin or *trans*-DDP (Sibghat-Ullah et al., 1989; Hansson and Wood,

1989). In contrast, extracts from XPA, XPC, XPD and XPG cells do not exhibit repair synthesis (Hansson and Wood, 1989), indicating that the signal observed in wild type cells was due to nucleotide excision repair. Moreover, wild type cells are more capable than XP cells of reactivating plasmid DNA that has been damaged with cisplatin (Poll et al., 1984; Chu and Berg, 1987; Maynard et al., 1989). Calsou *et al.* (Calsou et al., 1992) varied the relative levels of cisplatin adducts and determined that the 1,2-intrastrand crosslinks are poorly repaired by nucleotide excision repair and that the repair synthesis observed after incubation with globally modified plasmid DNA is due to excision of the minor adducts of cisplatin. The repair of individual DNA adducts formed by cisplatin has also been examined *in vitro* with an excision repair assay or a repair synthesis assay. Initial studies revealed that the 1,2-d(GpG) cisplatin adduct was refractory to excision repair (Szymkowski et al., 1992). Subsequent studies, however, demonstrated that the 1,2-d(GpG) adduct stimulates excision repair synthesis albeit 15-20 fold less efficiently than the 1,3-d(GpTpG) adduct (Moggs et al., 1997). Excision repair assays using both human cell extracts or a reconstituted excinuclease confirmed that the 1,3-d(GpTpG) adduct is more efficiently repaired than the 1,2-d(GpG) or 1,2-d(ApG) adduct (Huang et al., 1994; Zamble et al., 1996). No repair was detected for a cisplatin interstrand crosslink (Zamble et al., 1996).

Base Excision Repair

In contrast to nucleotide excision repair, which results in excision of an oligonucleotide containing the damaged site, base excision repair results in excision of just the damaged base. The first step in base excision repair is catalyzed by a class of enzymes called DNA glycosylases and involves the cleavage of the N-glycosydic bond between the damaged base and the deoxyribose

phosphate backbone, leaving behind an apurinic/apyrimidinic (AP) site. Bifunctional glycosylases possess and additional AP lyase activity that cleaves the phosphodiesterase backbone 3' to the AP site and leaves a modified 5' end at the nick, which is processed by the deoxyribophosphodiesterase or phosphatase activity of an AP endonuclease. Alternatively, the AP site is processed by an AP endonuclease, which hydrolyzes the phosphodiester bond 5' to the AP site; then, the deoxyribose phosphate residue is excised by a deoxyribophosphodiesterase. The resulting single-nucleotide gap is filled by a DNA polymerase, and a DNA ligase completes the repair reaction (Friedberg et al., 1995). Interestingly, in mammalian cells AP sites are processed via two alternative pathways; the short patch base excision repair described above or the long patch base excision repair. The short patch base excision repair pathway requires the action of DNA polymerase β and can be reconstituted *in vitro* using human uracil DNA glycosylase, human AP endonuclease (APE1 or HAP1), DNA polymerase β, X-ray cross complementation protein 1 (XRCC1), and DNA ligase I or III (Kubota et al., 1996). The long patch base excision repair pathway involves the activity of polymerase β or δ, flap endonuclease (FEN1), and PCNA (Matsumoto et al., 1994; Frosina et al., 1996; Klungland and Lindahl, 1997; Stucki et al., 1998; Fortini et al., 1998). Although the relative contribution of these two pathways has yet to be elucidated, one report demonstrated that the long patch base excision repair occurs at oxidized or reduced AP sites, while normal AP sites are processed by the short patch pathway (Klungland and Lindahl, 1997) and one report demonstrated that the type of DNA glycosylase that excises the lesion determines which pathway will be followed (Fortini et al., 1999).

The structure of uracil glycosylases (Savva and Pearl, 1995; Mol et al., 1995), T4

endonuclease V (Vassylyev et al., 1995), human 7,8-dihydro-8-oxoguanine glycosylase (Bruner et al., 2000), human 3-methyladenine glycosylase (Lau et al., 1998), and *E. coli* AlkA glycosylase (Hollis et al., 2000; Labahn et al., 1996; Yamagata et al., 1996) reveal a novel mechanism of substrate recognition. In the enzyme-substrate complex the DNA is distorted, so an unpaired nucleotide of the substrate is rotated out of its stacked position in the DNA helix and into the active site of the enzyme, thus enabling chemical reactions to occur on atomic centers that would have been otherwise inaccessible. The following model has been proposed for damage recognition by DNA glycosylases (Verdine and Bruner, 1997). Glycosylases, which have basic DNA recognition surfaces, track along the surface of DNA under the attractive influence of electrostatic forces. While scanning the surface of DNA, the glycosylase encounters a locally destabilized site containing a base lesion that is perhaps already in an extrahelical orientation. The protein then locks its active site onto the lesion and catalytic chemistry ensues. Damaged bases might be more susceptible than normal bases to being flipped out of the DNA double helix. Alternatively, a DNA glycosylase might flip nucleotides out of the double helix indiscriminately, but only those damaged sites that fit precisely into the substrate binding site of the enzyme would be subject to hydrolysis.

3-Methyladenine DNA Glycosylases

DNA glycosylases are an extensive family of enzymes, some of which show a very narrow substrate specificity, while others recognize a wide range of base derivatives. *E. coli* possesses two glycosylases that are involved in the repair of 3-methyladenine (3-mA) in DNA. The 3-methyladenine DNA glycosylase I, or Tag, encoded by the *tag* gene, is constitutively expressed and removes 3-mA and, to a lesser degree, 3-methylguanine (3-mG) from DNA (Thomas et al.,

1982; Bjelland et al., 1993; Bjelland and Seeberg, 1996). The 3-methyladenine DNA glycosylase II, or AlkA, is the product of the *alkA* gene and is induced during the adaptive response to alkylation damage. AlkA catalyzes the excision of a structurally diverse group of alkylated bases from DNA. It removes purines and pyrimidines damaged in the major or the minor groove as well as undamaged adenine, guanine, thymine and cytosine (Berdal et al., 1998). Specifically, AlkA catalyzes the excision of a variety of alkylated bases, including 3-mA, 7-methylguanine (7-mG), 7,8-dihydro-8-oxoguanine (8-oxoG), hypoxanthine (Hx), 1,N^6-ethenoadenine (εA), oxidized products of thymine, and adducts formed by nitrogen mustards (Thomas et al., 1982; Carter et al., 1988; Habraken and Ludlum, 1989; Habraken et al., 1991; Habraken et al., 1991; Matijasevic et al., 1992; Matijasevic et al., 1993; Bjelland et al., 1994; Saparbaev and Laval, 1994; Seeberg et al., 1995; Bjelland and Seeberg, 1996; Matijasevic et al., 1996; Mattes et al., 1996; Berdal et al., 1998). 3-Methyladenine DNA glycosylases identified in eukaryotic cells include the mouse (Aag) and the human (AAG, also known as MPG or ANPG) enzymes, which can remove 3-mA, 3-mG, and 7-mG from DNA (Samson et al., 1991; Chakravarti et al., 1991; O'Connor and Laval, 1991; Engelward et al., 1993). Additionally, mammalian 3-methyladenine DNA glycosylases remove Hx, possibly 8-oxoG, and bases with etheno substitutions (Singer et al., 1992; Matijasevic et al., 1993; Saparbaev and Laval, 1994; Dosanjh et al., 1994; Dosanjh et al., 1994; Saparbaev et al., 1995; Mattes et al., 1996; Berdal et al., 1998; Wyatt et al., 1999).

The "GO" repair system

The "GO" system is composed of three proteins, MutM, MutY and MutT, which are responsible for protecting *E. coli* cells from 8-oxoG mutagenesis (Michaels and Miller, 1992;

Grollman and Moriya, 1993). Primer extension reactions catalyzed by DNA polymerases demonstrate that adenine and cytosine can be incorporated opposite 8-oxoG *in vitro*. Interestingly, replicative DNA polymerases (polymerase α, polymerase δ and polymerase III) preferentially incorporate adenine over cytosine opposite 8-oxoG, whereas repair polymerases (polymerase β and polymerase I) preferentially incorporate cytosine opposite 8-oxoG (Shibutani et al., 1991). If left unrepaired, incorporation of adenine opposite 8-oxoG would lead to a G: C to T: A transversion mutation. Accordingly, *mutM, mutY* and *mutT* cells are mutators; i.e., they have an increased spontaneous mutation frequency (Cabrera et al., 1988; Nghiem et al., 1988).

The *mutT* gene maps at 2.5 min on the *E. coli* chromosome and it encodes for a 21 kDa protein (Akiyama et al., 1987; Bhatnagar and Bessman, 1988). The MutT protein is a nucleotide triphosphatase that hydrolyzes 8-oxodGTP to 8-oxodGMP (Bhatnagar and Bessman, 1988; Bhatnagar et al., 1991; Maki and Sekiguchi, 1992), thereby eliminating 8-oxodGTP from the dNTP pool. Polymerase III can incorporate 8-oxodGTP opposite adenine or guanine with equal efficiency (Maki and Sekiguchi, 1992; Cheng et al., 1992), and therefore it is an important contributor to mutagenesis. Accordingly, *mutT* strains are characterized by a high frequency of A:T to C:G mutations (Schaaper and Dunn, 1987).

The *mutM* gene maps at 81 min on the *E. coli* chromosome (Cabrera et al., 1988). Interestingly, *mutM* strains have a higher frequency of G: C to T: A mutations (Cabrera et al., 1988) and accumulate more 8-oxoG in their DNA than wild type cells (Bessho et al., 1992; Wojcik et al., 1996). The *mutM* gene is the same as the *fpg* gene that has been independently cloned (Chetsanga

and Lindahl, 1979; Boiteux et al., 1987; Michaels et al., 1991). The *fpg* gene product, formamidopyrimidine DNA glycosylase (Fapy glycosylase or MutM) is a 30.2 kDa DNA glycosylase that efficiently removes 8-oxoG when it is paired with C (Chung et al., 1991; Tchou et al., 1991). The enzyme is much less efficient at binding to A: 8-oxoG mismatches and removing 8-oxoG from these mismatches (Michaels et al., 1992). MutM is a bifunctional DNA glycosylase; in addition to N-glycosylase activity, it has AP lyase activity that effects strand scission at AP sites via a β,δ-elimination reaction (Boiteux et al., 1990). Moreover, it has deoxyribophosphodiesterase activity that removes 5' terminal deoxyribosephosphate residues (Graves et al., 1992).

The *mutY* gene maps at 64 min on the *E. coli* chromosome (Nghiem et al., 1988), and it encodes a 39.1 kDa glycosylase that can excise adenine from A: 8-oxoG mismatches (Au et al., 1988; Au et al., 1989; Michaels et al., 1990). Interestingly, *mutY* strains also have a higher frequency of G: C to T:A mutations (Nghiem et al., 1988), and *mutM* and *mutY* strains have similar mutation spectra in the *lacI* forward mutation system (Cabrera et al., 1988; Nghiem et al., 1988). Moreover, overexpression of MutM can rescue the mutator phenotype of *mutY* strains (Michaels et al., 1992), and the *mutM mutY* double mutant has a 20 fold higher mutation frequency than the sum of either mutant alone (Michaels et al., 1992), indicating that these proteins function together in preventing G: C to T: A transversion mutations.

The following model has been proposed for the role of MutM and MutY proteins in preventing cells from mutagenesis by 8-oxoG lesions that can arise in DNA after oxidative damage (Michaels et al., 1992; Michaels et al., 1992; Michaels and Miller, 1992). Prior to DNA replication,

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MutM can remove 8-oxoG from DNA and restore the original G: C base pair. During replication

an adenine residue is preferentially incorporated opposite the 8-oxoG in DNA, generating a substrate

for MutY. After MutY excises the misincorporated adenine, it remains bound to the reaction product

until the duplex is repaired to 8-oxoG: C by the concerted activity of AP endonuclease, DNA

polymerase I and DNA ligase. MutY binding to the duplex containing the 8-oxoG: AP base pair

prevents its recognition by MutM. Recognition of this base pair by MutM, followed by excision of

8-oxoG, would generate a double strand break, which is toxic to cells. Once the 8-oxoG: C is

restored, MutM gets another chance at removing 8-oxoG from DNA. Interestingly, the spontaneous

mutation frequency of *mutY* cells is higher than that of the *mutM* cells, suggesting that MutY is the

dominant mechanism for dealing with 8-oxoG produced in DNA. Accordingly, 8-oxoG is more

mutagenic in a *mutY* strain than in a wild type strain when located in a single stranded vector

(Moriya and Grollman, 1993), but not when situated in a double stranded vector (Wagner et al.,

1997). Moreover, the mutagenesis of a single 8-oxoG situated in a single stranded (Moriya and

Grollman, 1993) or a double stranded (Wagner et al., 1997) vector is increased in a *mutM mutY*

strain compared to the *mutM*, *mutY*, or wild type strains.

MutM

Interestingly, oxidative stress generated by hydrogen peroxide treatment increases the

frequency of short homology dependent illegitimate recombination. MutM, but not MutY or MutT,

can suppress these recombination events, possibly by repairing the hydrogen peroxide generated

damage that induces illegitimate recombination (Onda et al., 1999).

In addition to its role in 8-oxoG repair, the MutM protein catalyzes the excision or ring opened purines. In particular it can excise 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine, N2-aminofluorene-C8-guanine, aflatoxin B1 FAPY adducts, and adducts formed in DNA after treatment with 7-hydroxyethyl-thioethylguanine. Finally, 7,8-dihydro-8-oxo-2'-deoxyadenosine (8-oxoA), 7,8-dihydro-8-oxo-2'-deoxyinosine, 7,8-dihydro-8-oxo-2'-deoxynebularine, 5-hydroxycytosine, and 5-hydroxyuracil can also be released by MutM (Chetsanga and Lindahl, 1979; Chetsanga et al., 1982; Chetsanga and Frenette, 1983; Breimer, 1984; O'Connor et al., 1988; Boiteux et al., 1989; Laval et al., 1990; Boiteux et al., 1990; Chung et al., 1991; Tchou et al., 1991; Boiteux et al., 1992; Graves et al., 1992; Oleykowski et al., 1993; Li et al., 1997; Karakaya et al., 1997).

MutY

Biochemical experiments indicate that MutY removes adenine from A: G, A: 8-oxoG, A: C, and A: 8-oxoA mismatches (Lu and Chang, 1988; Au et al., 1989; Michaels et al., 1992; Michaels et al., 1992; Tsai-Wu et al., 1992). Moreover, MutY removes guanine from G: 8-oxoG mismatches (Zhang et al., 1998; Li et al., 2000), but it has no activity on C: 8-oxoG or T: 8-oxoG mismatches (Michaels et al., 1992). In addition MutY cannot remove 8-oxoG or 8-oxoA from any of the mismatches (Michaels et al., 1992). Experiments with heterozygous bacteriophage λ molecules have demonstrated that *mutY* strains cannot remove adenine from A: C or A: G mismatches *in vivo* (Radicella et al., 1988). The importance of MutY activity on preventing mutagenesis is underscored by the observation that *mutY* strains have an increased frequency of G: C to T: A and G: C to C: G transversion mutations (Nghiem et al., 1988; Zhang et al., 1998), consistent with the inability of this

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strains to repair A: 8-oxoG and G: 8-oxoG mismatches.

To date there is lack of agreement on whether to classify MutY as a monofunctional or a bifunctional DNA glycosylase. Several groups have failed to detect AP lyase activity for MutY (Au et al., 1989; Michaels et al., 1992; Michaels et al., 1992; Sun et al., 1995; Bulychev et al., 1996), whereas other groups have reported the activity to be present (Tsai-Wu et al., 1992; Lu et al., 1995; Gogos et al., 1996; Lu et al., 1996; Manuel et al., 1996; Manuel and Lloyd, 1997). MutY is capable of cleaving DNA containing an AP site (Manuel and Lloyd, 1997; Wright et al., 1999), and it can form a covalent Schiff base intermediate with substrate DNA (Lu et al., 1996; Williams and David, 1998; Zharkov and Grollman, 1998; Williams and David, 1999; Wright et al., 1999). The formation of such a Schiff base intermediate can be detected by borohydride reduction of the imine generated during the strand scission reaction and is routinely used as a diagnostic tool for bifunctional DNA glycosylases. Studies with site specific mutants of MutY (Williams and David, 1999; Wright et al., 1999), as well as Edman degradation of the covalent complex (Zharkov and Grollman, 1998) indicate that the Lys 142 of MutY is involved in the Schiff base formation. Importantly, K142A mutants can still catalyze excision of adenine from A: G and A: 8-oxoG substrates (Williams and David, 1999; Wright et al., 1999) and can cleave AP site containing DNA (Wright et al., 1999), indicating that Schiff base formation is not required for the catalytic activity of MutY. Comparison of the rate of N-glycosylase activity with the rate of strand scission at AP sites reveals that the strand scission reaction takes place at a much slower rate that the glycosylase reaction (Williams and David, 1998; Wright et al., 1999). Furthermore, Sugiyama *et al.* (Sugiyama et al., 1994) demonstrated that under heating conditions, AP sites selectively decompose to 3' termini possessing an α,β-unsaturated

aldose residue and a 5' phosphate residue, and Zharkov and Grollman (Zharkov and Grollman, 1998) suggested that the AP lyase activity of MutY detected by some groups is actually strand scission effected by heat. However, subsequent studies demonstrated strand scission at AP sites in the absence of heat (Wright et al., 1999). In conclusion MutY has a weak AP lyase activity and the lyase reaction does not take place in a concerted fashion with the glycosylase reaction.

Several studies have compared the kinetics of adenine removal from A: 8-oxoG and A: G mismatches. Some groups present evidence that A: G mismatches are the preferred substrates for MutY (Lu et al., 1996), whereas other groups claim that the opposite is true (Bulychev et al., 1996). Porello et al. (Porello et al., 1998) demonstrated that monitoring the adenine excision from A: G or A: 8-oxoG mismatches under multiple turnover conditions ([MutY]<<[Substrate]) yields different results at different time points. Analysis of the reaction products after a short period of time indicates that A: 8-oxoG substrates are more reactive than A: G substrates, whereas at longer times more product is observed with the A: G substrate. This is because MutY can have multiple catalytic turnovers with the A: G substrate, while with A: 8-oxoG it remains bound to product. Hence MutY is unable to process any additional substrate beyond the first turnover (Porello et al., 1998). These results indicate that glycosylase activity assays with MutY would be influenced by the relative amount of MutY to substrate, and under these conditions the Michaelis-Menten kinetic parameters K_{cat} and K_M cannot be determined. Thus, the activity of MutY on A: G and A: 8-oxoG mismatches cannot be compared bu using the K_{cat} and K_M parameters reported. Interestingly, under single turnover conditions ([MutY]>>[Substrate]), the rate of adenine excision from an A: 8-oxoG mismatch is greater than that for the A: G mismatch (Porello et al., 1998), indicating that MutY is

intrinsically more active with the A: 8-oxoG substrate.

The binding affinity of MutY for A: G and A: 8-oxoG containing substrates has also been determined. The dissociation constant for the A: G substrate ranged from 5.3 nM to 35 nM, whereas that for an A: 8-oxoG substrate ranged from 66 pM to 5.5 nM (Bulychev et al., 1996; Lu et al., 1996; Porello et al., 1996). Because of catalytic turnover, these numbers reflect the binding affinity for a mixture of substrate and product. Therefore, the values are very sensitive to the composition of the mixture, which depends on the reaction conditions. When a catalytically inactive mutant of MutY, D138N, was used in binding assays the dissociation constant for the A: 8-oxoG substrate increased 1.5 fold, whereas that for the A: G substrate increased 4.5 fold (Wright et al., 1999).

A number of adenine and guanine analogues have been used in binding and glycosylase activity assays in order to determine the structural requirements for MutY recognition and enzymatic activity. MutY retains glycosylase activity with substrates where 8-oxoG has been substituted with 8-methoxy-deoxyguanosine or inosine, as well as with substrates where A has been substituted with inosine or 2-aminopurine (Lu et al., 1995; Bulychev et al., 1996). Moreover, MutY binds with fairly high affinity to substrates containing structural analogues of adenine, such as 7-deaza-deoxyadenosine (Z), deoxyformycin A (F), deoxy-2'-fluoroadenosine (FA), or deoxyaristeromycin (R) opposite 8-oxoG or G but it shows no excision activity with any of the duplexes (Bulychev et al., 1996; Porello et al., 1996; Chepanoske et al., 1999). Therefore, these substrates can be used to investigate the properties of substrate recognition without the complication of catalytic turnover. Indeed, comparison of the binding affinities of MutY for DNA substrates containing G: C, G: F,

8-oxoG: C, or 8-oxoG: F base pairs indicates that MutY recognizes both bases of the mismatch in a synergistic manner. This result is consistent with the results from footprinting studies; MutY protects an area of 8 nucleotides of the 8-oxoG containing strand and an area of 8 nucleotides in the F (or FA) containing strand of an 8-oxoG: F (or 8-oxoG: FA) substrate from hydroxyl radical cleavage (Porello et al., 1996; Chepanoske et al., 1999), suggesting that MutY makes contact with both strands. Moreover, MutY binding to the product of the glycosylase reaction with an A: 8-oxoG substrate protects 8 nucleotides surrounding the 8-oxoG lesion (Porello et al., 1996), thereby providing the structural basis for the inhibition of MutM binding to an 8-oxoG: AP substrate in the presence of MutY. The contacts of MutY with the 8-oxoG containing strand are thought to be important in promoting extrusion of the misincorporated base into the adenine specific pocket of MutY. Furthermore, a guanine residue in the strand containing the substrate analogues R, F or FA becomes hypersensitive to DMS upon MutY binding (Chepanoske et al., 1999). This result indicates that binding of MutY to duplex mismatched DNA results in a conformational change of the substrate that exposes the substrate analogue-containing strand. Finally, ethylation and methylation intereference experiments, as well as DMS footprinting experiments with A: 8-oxoG or A: G containing substrates, confirmed that MutY has contacts with the 8-oxoG (or G) containing strand and demonstrated that MutY has contacts in both the major and minor grooves (Lu et al., 1995; Chepanoske et al., 1999).

Trypsin or thermolysin digestion identified two distinct structural domains for MutY (Gogos et al., 1996; Manuel et al., 1996). The small, 13 kDa C-terminal domain is evolutionarily related to MutT and is thought to confer specificity for A: 8-oxoG mismatches (Gogos et al., 1996; Noll et al.,

1999; Li et al., 2000). The large, N-terminal 26 kDa domain is called p26 and has similar substrate specificity to that of the full length protein; p26 recognizes A: G and A: 8-oxoG mismatches (Gogos et al., 1996; Manuel and Lloyd, 1997; Li et al., 2000). Furthermore, the p26 domain retains the ability to excise adenine when paired with guanine or 8-oxoG (Gogos et al., 1996; Manuel et al., 1996; Manuel and Lloyd, 1997; Noll et al., 1999; Li et al., 2000). A site directed D138N mutant p26 can still bind to DNA containing A: G and A: 8-oxoG mismatches, albeit with slightly lower affinity than wild type enzyme. However, this mutant has lost the ability to remove adenine from A: G and A: 8-oxoG mismatches (Guan et al., 1998).

The crystal structure of the catalytically competent p26 domain of MutY with bound adenine has been solved (Guan et al., 1998). The structure suggests that the mismatched adenine of the A: 8-oxoG or A: G mismatch rotates out of the DNA helix and binds in a specificity pocket in MutY formed by conserved residues Gln 182, Glu 37, and Asp 186, and tightly packs between the hydrophobic residues Leu 40 and Met 185. Based on the crystal structure and studies with substrate analogues and site specific mutants of MutY, the following model for enzymatic catalysis by MutY has been proposed. Upon encountering an 8-oxoG: A mismatch, MutY causes the mismatched adenine to be rotated out of the DNA helix and into the enzyme active site. The Asp 138 residue in the active site is positioned to abstract a proton from a nearby water molecule, thereby activating the water molecule for nucleophilic attack of the C1' of the target adenine, while a protonated Glu 37 is positioned to stabilize the developing transition state through a hydrogen bond to the N7 of adenine.

The role of the Fe-S cluster of MutY on enzymatic activity has also been investigated. Tsai Wu et al. (Tsai-Wu et al., 1992) reported that enzymatic activity of MutY can be recovered if the protein is renatured in the presence of ferrous iron and sulfide, providing the first indication that MutY is an iron sulfur protein. Further studies confirmed that MutY contains a $[4Fe-4S]^{2+}$ cluster and showed that *in vitro* assembly of this cluster is not required for folding or thermal stability of MutY *in vitro* (Porello et al., 1998). Mutations in residues coordinated to the $[4Fe-4S]^{2+}$ cluster reduce the levels of protein expression, indicating that the cluster contributes to MutY stability *in vivo* (Golinelli et al., 1999). Moreover, the presence of the cluster is required for binding and excision activity (Porello et al., 1998). It is important to note that MutY protein folded in the absence of ferrous and sulfide ions regains activity after addition of these ions in the mixture indicating a superficial location of the cluster (Porello et al., 1998). Indeed, the crystal structure of MutY indicated that the $[4Fe-4S]^{2+}$ cluster is located in the region that interacts with DNA (Guan et al., 1998).

The human homologue of the *mutY* gene (*hMYH*) has been cloned and sequenced (Slupska et al., 1996). hMYH is a 59 kDa protein that shows 41% identity to the *E. coli* MutY protein. Moreover, hMYH can remove adenine from A: 8-oxoG and A: G mismatches and after prolonged incubation it can remove guanine from G: 8-oxoG mismatches (McGoldrick et al., 1995; Slupska et al., 1999). Finally, expression of *hMYH* in *mutY* cells rescues their mutator phenotype (Slupska et al., 1999), suggesting that hMYH has the same properties as MutY.

Mismatch Repair

Mismatch repair is a specialized correction mechanism for single base or insertion/deletion mismatches that arise in DNA during replication. The *E. coli* system is very efficient at repairing G: C and A: C mismatches. The G: G and A: A mismatches are usually corrected efficiently, the T: T, C: T and G: A mismatches are corrected less efficiently, the C: C mismatches are not usually corrected by mismatch repair. Moreover, while 1-, 2-, or 3-base loops are efficiently repaired, 4-base loops are marginally repaired, and 5-base loops are not repaired by this system. The *E. coli* mismatch repair system uses the transient undermethylation of the daughter strand immediately after DNA replication in order to distinguish the daughter from the parental strand and directs repair to the daughter strand. In particular, it detects the state of N^6 methylation of adenine at GATC sites Methylation is catalyzed by dam methylase and the differential state of methylation distinguishes recently synthesized (unmethylated) from parental (methylated) DNA strands. The repair reaction has been reconstituted *in vitro* with MutS, MutL, MutH, UvrD, single stranded binding protein, polymerase III holoenzyme, exonuclease I, exonuclease VI, RecJ, DNA ligase, ATP, all four dNTPs, and NAD^+, and the following model has been proposed. Repair initiates by binding of a MutS dimer to the mismatch. A MuL dimer binds to this complex and stimulates a latent endonuclease activity of MutH that cleaves DNA at the GATC sequence of the unmethylated strand. The nick directs excision of a long DNA patch (up to 10^3 bases) containing the incorrect base, followed by a filling in of the gap and ligation (Friedberg et al., 1995). Mismatch repair has been implicated in the cellular responses of *E. coli* cells to methylating agents and cisplatin. Several studies have shown that *dam* cells are more sensitive to MNNG and cisplatin, and introduction of a *mutS* or a *mutL*, but not a *mutH* mutation, in these cells abrogates hypersensitivity to these agents (Jones and Wagner,

1981; Karran and Marinus, 1982; Fram et al., 1985).

The importance of mismatch repair in maintaining genomic integrity is underscored by the finding that humans carrying mutations in some homologues of the mismatch repair genes are susceptible to cancer. In particular 70% of all hereditary non polyposis colon cancer cases are associated with a deficiency in the mismatch repair genes. The mechanism of mismatch repair in human cells is not well understood and it appears to be more complex than the one in *E. coli*. The strand discrimination signal in mammalian cells is unknown, but a nick in one strand of the DNA, such as would occur in Okazaki fragments, is sufficient to direct mismatch repair to that strand *in vitro* (Holmes et al., 1990; Thomas et al., 1991). Mismatch recognition occurs with one of two heterodimers of MutS homologues. hMutSα (heterodimer of hMSH2 and hMSH6) binds to mismatches and small insertion/deletion loops, whereas hMutSβ (heterodimer of hMSH2 and hMSH3) recognizes larger insertion/deletion loops (Bhattacharyya et al., 1994; Drummond et al., 1995; Marsischky et al., 1996; Palombo et al., 1996; Acharya et al., 1996). A heterodimer of MutL homologues, hMutLα (hMLH1 and hPMS2 heterodimer), binds to this complex. Moreover, hMutLα can interact with PCNA, suggesting that a higher order complex is assembled. A second heterodimer of MutL, hMutLβ (hMLH1 and hPMS1 heterodimer), has been identified but is thought to play a minor role in mismatch (Kramer et al., 1989; Prolla et al., 1994; Li and Modrich, 1995; Flores-Rozas and Kolodner, 1998; Prolla et al., 1998).

Interestingly, MNNG resistant cell lines isolated after prolonged exposure to the drug appear to have acquired mutations in one of the mismatch repair genes and, conversely, mismatch repair

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deficient cell lines are resistant to alkylation damage (Goldmacher et al., 1986; Kat et al., 1993; Branch et al., 1993; Koi et al., 1994; Papadopoulos et al., 1995; de Wind et al., 1995; Aquilina et al., 1995; Branch et al., 1995; Hampson et al., 1997; Umar et al., 1997). Similar results have been observed in studies with cisplatin. Cisplatin resistant cell lines isolated after exposure to the drug *in vitro* have acquired the mutator phenotype associated with mismatch repair defects and are usually *hMLH1* deficient (Anthoney et al., 1996; Aebi et al., 1996; Drummond et al., 1996; Brown et al., 1997a). Moreover, a screening of ovarian tumor samples prior to and after platinum based chemotherapy revealed a significant increase in the proportion of tumors deficient in hMLH1 expression after the treatment (Brown et al., 1997a). These studies indicate that exposure of cells to cisplatin can result in loss of mismatch repair. It in unclear, however, whether cisplatin treatment causes inactivation of the mismatch repair genes, or whether it selects for cells that are already deficient in mismatch repair. A study with a mixed population of wild type and mismatch repair deficient mouse cells demonstrated that cisplatin treatment results in an enrichment of the repair deficient population, suggesting that at least the latter mechanism can be operative *in vivo* (Fink et al., 1997; Fink et al., 1998; Fink et al., 1998). The importance of mismatch repair in clinical response was evaluated in a study that measured hMSH2 and hMLH1 expression in paired tumors of ovarian cancer patients obtained before and after platinum based chemotherapy (Samimi et al., 2000). hMSH2 and hMLH1 expression decreased significantly after treatment, consistent with the ability of cisplatin treatment to select for mismatch repair deficient cells in an animal model. However, no association between the expression of either protein and overall survival was apparent, suggesting that hMSH2 and hMLH1 levels are not predictive of clinical response.

Complementary studies with mismatch repair deficient cells have shown that inactivation of mismatch repair genes confers resistance to cisplatin. In particular the colorectal cancer cell line HCT116, which is deficient in *hMLH1*, the colorectal cancer cell line DLD1, which is deficient in *hMSH6*, and the endometrial cancer cell line HEC59, which is deficient in *hMSH2*, are up to four fold more resistant to cisplatin than the corresponding sublines that have been corrected for the repair defect by chromosomal transfer (Aebi et al., 1996; Fink et al., 1996; Vaisman et al., 1998). Moreover, the HHUA colorectal cancer cell line that is deficient in *hMSH3* and *hMSH6* becomes sensitive to cisplatin when corrected for the *hMSH6* deficiency but not when corrected for the *hMSH3* deficiency by chromosomal transfer (Vaisman et al., 1998). Further studies with mouse embryonic stem cells or immortalized mouse fibroblasts indicate that $MSH2^{-/-}$ cells are two fold more resistant than $MSH2^{+/+}$ cells (Fink et al., 1997; Reitmair et al., 1997). This difference in response may have clinical significance, since xenograft tumors in mice generated from $MSH2^{-/-}$ cells are less responsive to cisplatin than those generated by $MSH2^{+/+}$ cells (Fink et al., 1997). Interestingly, the mismatch repair deficient cells are also resistant to the clinically effective cisplatin analog carboplatin, but not to oxaliplatin or to the clinically ineffective *trans*-DDP (Aebi et al., 1996; Fink et al., 1996). Finally, mismatch repair deficient cell lines are resistant to MNNG or cisplatin induced chromosomal rearrangements, G2/M cell cycle arrest and apoptosis (Goldmacher et al., 1986; Hawn et al., 1995; Brown et al., 1997b).

Recombinational Repair

There are two pathways for recombinational repair in *E. coli*. The daugther strand gap repair pathway requires the activity of RecA, RecF, RecO, RecR, RuvA, RuvB, RuvC and RecG. The

double strand break repair pathway requires the activity of RecA, RecB, RecC, RecD, RuvA, RuvB, RuvC and RecG. The importance of recombination in the survival of cisplatin treated cells was first observed by Beck in 1973 (Beck and Brubaker, 1973), who demonstrated that *recA, recB* and *recC* cells are more sensitive to cisplatin than wild type cells, and *recB recC* cells are more sensitive than either mutant alone. Further studies showed that *recA* and *recB* cells are more sensitive to cisplatin than wild type cells (Alazard et al., 1982). Moreover, *uvrA recA* cells are more sensitive than either mutant alone, indicating that the role of RecA in protecting cells from cisplatin toxicity is independent of nucleotide excision repair (Alazard et al., 1982). Husain *et al.* (Husain et al., 1985) determined the ability of *uvrA, recA, uvrA recA*, and wild type cells to reactivate plasmid DNA damaged with the cisplatin analogue DACH. Cells carrying mutations in *recA* locus repair damaged DNA less efficiently than *recA*$^+$ cells regardless of *uvrA* status. Moreover, the *uvrA* gene appeared to play a bigger role in the repair of the damaged DNA than the *recA* gene.

A recent study analyzed in detail the sensitivity of various recombination deficient mutants to cisplatin and *trans*-DDP (Zdraveski et al., 2000). Almost all of the strains tested are very sensitive to cisplatin, whereas they display modest sensitivity to *trans*-DDP. *recF, recO,* and *recR* cells, which are deficient in daughter strand gap repair, and *recB recC,* but not *recD* cells, which are deficient in double strand break repair aremore sensitive to cisplatin than the corresponding wild type cells. In addition mutants deficient in resolving Holliday junctions (*ruvA, ruvC, recG,* and *ruvC recG*) are also sensitive to cisplatin. Moreover, mutants deficient in both nucleotide excision repair and recombinational repair (*recF uvrA, recB recC recD uvrA, ruvA uvrA,* and *ruvC uvrA*) are more sensitive to cisplatin than the single mutants, providing further support for the notion that the role

of recombination proteins in protecting cells from cisplatin toxicity is independent of nucleotide excision repair.

The importance of recombination in the repair of cisplatin interstrand crosslinks was recently evaluated in yeast. *S. cerevisiae* strains deficient in recombination, non homologous end joining, or mutagenesis pathways demonstrated that all are essential in the repair of cisplatin interstrand crosslinks, and the relative importance of these pathways is cell cycle dependent (McHugh et al., 2000).

E. Proteins Recognizing Cisplatin Adducts

It has been suggested that the difference in clinical efficacy of cisplatin and *trans*-DDP arises from the distinct structural alterations induced in DNA after binding of these drugs and the differential recognition and processing of the adducts by cellular proteins. Thus, significant effort has been put into identifying cellular proteins that recognize specifically adducts of clinically effective platinum drugs.

Photolyase

Photolyase is a flavoprotein that uses near UV or visible light (300 to 500 nm) to reverse the cis-syn pyrimidine dimers produced in DNA after UV irradiation. Moreover, in the absence of photoreactivating light, photolyase binds to the pyrimidine dimers and stimulates their repair by the Uvr(A)BC excinuclease (Sancar et al., 1984). The structure of photolyase suggests that the pyrimidine dimer is rotated out of the DNA helix and into the enzyme active site (Vande and Sancar,

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1998). Interestingly, *E. coli* cells expressing photolyase are more resistant to cisplatin treatment than photolyase deficient cells (Ozer et al., 1995), suggesting that photolyase might be involved in the repair of cisplatin adducts. Accordingly, the *E. coli* photolyase binds to duplex DNA containing a single 1,2-d(GpG) adduct with high affinity (K_d = 50 nM), and stimulates *in vitro* repair of this adduct by the Uvr(A)BC excinuclease (Ozer et al., 1995). Since photolyase bends the DNA helix by 36° when bound to UV damaged sites (van Noort et al., 1999), it is likely that photolyase recognizes the bending in the DNA induced by the cisplatin adducts.

The DNA binding surface of the *S. cerevisiae* photolyase has 50% identity with that of the *E. coli* enzyme. Accordingly, yeast photolyase recognizes cisplatin adducts but has no affinity for DNA modified with *trans*-DDP (Fox et al., 1994). Interestingly, *S. cerevisiae* cells deficient in photolyase are more resistant to cisplatin than wild type cells (Fox et al., 1994), and it has been suggested that this enzyme binds to cisplatin adducts and shields them from repair. These cells were not differentially sensitive to *trans*-DDP, consistent with the lack of affinity for *trans*-DDP adducts (Fox et al., 1994).

XPE

XP group E is the mildest form of the XP disorder, and XPE deficient cells generally show 50% of the normal repair level. Even though the exact function of XPE is unknown, it is thought to participate in damage recognition. Specifically, it has been suggested that XPE is the human homologue of the *S. cerevisiae* photolyase (Patterson and Chu, 1989). Human cell extracts contain a factor that binds specifically to cisplatin damaged DNA, and this factor is absent in *XPE* deficient

cells (Chu and Chang, 1988). Moreover, purified XPE protein recognizes cisplatin adducts, whereas it has no affinity for *trans*-DDP adducts (Payne and Chu, 1994). Interestingly, XPE expression is induced after treatment with cisplatin (Vaisman and Chaney, 1995), and human tumor cell lines selected for resistance to cisplatin show more efficient DNA repair and increased expression of XPE (Chu and Chang, 1990). Although it is very tempting to attribute the increased repair of cisplatin adducts to the higher levels of XPE, it is important to remember that DNA repair is a sequential multienzyme process and unless a protein is involved in the rate limiting step, its overexpression would not affect the overall levels of repair. This point is underscored by the finding that microinjection of XPE protein in *XPE* deficient cells restores UV damage repair to wild type levels but injection of higher levels gives no further stimulation of the repair activity (Keeney et al., 1994).

Nucleotide Excision Repair Proteins

XP group A is one of the most frequently encountered forms of XP. The XPA protein is involved in the damage recognition step of nucleotide excision repair and it can interact with ERCC1, the p34 subunit of RPA and TFIIH. Both the DNA recognition domain of XPA and the full length protein have higher affinity for cisplatin damaged DNA than unmodified DNA (Jones and Wood, 1993; Asahina et al., 1994; Kuraoka et al., 1996), and the XPA protein can be photocrosslinked to DNA containing a single 1,3-d(GpTpG) adduct (Schweizer et al., 1999). Moreover, *XPA* deficient cells are more sensitive to cisplatin than wild type cells (Fraval et al., 1978; Poll et al., 1984; Dijt et al., 1988) and enhanced expression of XPA mRNA is observed in tumor tissues from ovarian cancer patients that are resistant to platinum based chemotherapy compared to levels in tissues of patients that responded favorably to chemotherapy (Dabholkar et al., 1994; Ferry

et al., 1996).

Interestingly the interaction of XPA with ERCC1 increases the binding affinity of XPA for UV damaged DNA (Nagai et al., 1995), indicating that ERCC1 might also be involved in the damage recognition step of nucleotide excision repair. Moreover, there is a statistically significant correlation between the relative expression of *XPA* and *ERCC1* mRNAs in ovarian tumors (Codegoni et al., 1997). Accordingly, *ERCC1* mRNA levels correlate with response to platinum based chemotherapy with the higher mRNA levels being observed in tumors refractory to chemotherapy (Dabholkar et al., 1992; Dabholkar et al., 1994; Metzger et al., 1996).

RPA (also known as single stranded DNA binding protein) is a heterotrimeric protein composed of p70, p24 and p14 subunits, and it is involved in DNA replication, repair and homologous recombination. Even though RPA can bind to damaged DNA alone, it has been implicated in stabilizing the opened DNA duplex in cooperation with XPA, TFIIH, and XPC. Moreover, RPA interacts with XPA, XPG, and XPF-ERCC1, indicating that it is involved in the damage recognition and excision steps of nucleotide excision repair. Interestingly, RPA has been identified in cisplatin damaged DNA protein complexes by Western blot analysis (Clugston et al., 1992), and purified RPA binds with higher affinity to cisplatin damaged DNA than unmodified DNA (Patrick and Turchi, 1998; Patrick and Turchi, 1999; Schweizer et al., 1999). Moreover, it recognizes a single 1,2-d(GpG) and a single 1,3-d(GpTpG) adduct (Patrick and Turchi, 1999), and the relative binding affinities of RPA to the different cisplatin adducts correlate with the repair of the adducts observed in an *in vitro* repair assay (Zamble et al., 1996); RPA has a higher affinity for

the 1,3-d(GpTpG) cisplatin adduct than for the 1,2-d(GpG) adduct. A DNA substrate containing a single cisplatin interstrand crosslink is poorly recognized (Patrick and Turchi, 1999).

Interestingly, the binding affinity of an XPA-RPA complex for a DNA substrate containing a single 1,3-d(GpTpG) adduct is greater than that of RPA (Schweizer et al., 1999). Furthermore, photocrosslinking studies demonstrated that the p70 subunit of RPA can be crosslinked with high efficiency to DNA containing a single 1,3-d(GpTpG) adduct (Schweizer et al., 1999). Moreover, when RPA and XPA are both present in the reactions, only the RPA protein gets crosslinked to DNA (Schweizer et al., 1999). These results suggest that the RPA protein plays a major role in cisplatin adduct recognition.

Interestingly, the amount of RPA binding to cisplatin modified DNA correlates with the ability of the protein to denature DNA (Patrick and Turchi, 1999). Moreover, RPA binds with higher affinity to unmodified single stranded DNA than to DNA containing a single 1,2-d(GpG) cisplatin adduct (Patrick and Turchi, 1999). These results indicate that RPA binds to duplex DNA, causes denaturation of the DNA helix, and then binds preferentially to the undamaged strand. Previous studies have demonstrated that RPA can enhance the binding and excision activities of XPG and XPF-ERCC1 to bubble and loop substrates (Matsunaga et al., 1996), and it has been suggested that the protein protects the undamaged strand from excision (Evans et al., 1997; de Laat et al., 1998). The preferential binding of RPA to the unmodified strand provides a structural basis for the direction of excision repair to the damaged strand.

Mismatch Repair Proteins

Mismatch repair proteins are expressed in high levels in testicular tissues. Specifically, hMSH2 protein levels are increased in testicular and ovarian tissues (Mello et al., 1996). Testicular tissues also have higher mRNA levels of *hMSH2*, *hMLH1*, *hMSH4*, and *hMSH5* (Wilson et al., 1995; Fink et al., 1997; Paquis-Flucklinger et al., 1997; Winand et al., 1998; Her and Doggett, 1998). hMSH2 binds to DNA containing an average of 6 cisplatin adducts with high affinity (K_d = 67 nM), and it can recognize specifically DNA containing a single 1,2-d(GpG) adduct (Mello et al., 1996). Moreover, hMSH2 displays selective affinity for adducts of clinically effective platinum drugs; it recognizes cisplatin and Pt(en)Cl$_2$ adducts, but not *trans*-DDP or [Pt(dien)Cl]$^+$ adducts (Mello et al., 1996). Additionally, the hMutSα heterodimer recognizes the 1,2-d(GpG) cisplatin adduct, but it has no affinity for the 1,3-d(GpTpG) adduct of *trans*-DDP (Duckett et al., 1996). The binding affinity of hMutSα for the 1,2-d(GpG) adduct is enhanced in duplexes containing a thymine incorporated opposite the 5' or the 3' guanine of the adduct (Yamada et al., 1997; Mu et al., 1997). As mentioned above deficiencies in mismatch repair genes are associated with cisplatin resistance (Aebi et al., 1996; Fink et al., 1996; Vaisman et al., 1998). Accordingly, the following model for the role of mismatch repair in cisplatin toxicity has been proposed. Mismatch repair proteins recognize cisplatin adducts in the template strand, and they then attempt to repair the "damage" in the newly synthesized strand. Since there is no correct base that can be incorporated opposite the cisplatin adduct, a futile cycle of repair is initiated. This recurrent misdirected repair could generate a cell cycle arrest and/or an apoptotic signal. Moreover, mismatch repair proteins might trigger cell cycle arrest and/or apoptosis through the activation of the JNK or c-Abl signaling pathways (Nehme et al., 1997; Gong et al., 1999; Nehme et al., 1999). It was originally proposed that mismatch repair

proteins might mediate cisplatin toxicity by shielding cisplatin adducts from repair, but this model was refuted by studies that determined that nucleotide excision repair activity is unaffected by the presence of hMutSα (Moggs et al., 1997; Mu et al., 1997). Alternatively, mismatch repair proteins might bind to cisplatin adducts and inhibit replication bypass (see below).

T4 endonuclease VII

T4 endonuclease VII is an enzyme that resolves branched DNA structures. In particular it is very efficient at cleaving four way junctions, three way junctions and looped DNA structures. Interestingly, T4 endonuclease VII binds to and cleaves substrate DNA containing a single 1,2-d(GpG) or 1,2-d(ApG) adduct (Murchie and Lilley, 1993). Moreover, the 1,2-d(GpG) adduct is a better substrate for this enzyme than the 1,2-d(ApG) adduct (Murchie and Lilley, 1993), indicating that these adducts cause different alterations in the DNA architecture. In contrast 1,3-d(GpTpG) adducts of *trans*-DDP are not substrates for T4 endonuclease VII (Murchie and Lilley, 1993). Furthermore, T4 endonuclease VII can cleave interstrand crosslinks of cisplatin and *trans*-DDP, but the interstrand crosslink of cisplatin is cleaved more efficiently than the interstrand crosslink of *trans*-DDP (Kasparkova and Brabec, 1995).

Human Ku autoantigen

Ku is a DNA binding protein with affinity for ends of double stranded DNA and DNA substrates containing small gaps and nicks. The Ku-DNA complex stimulates the catalytic subunit of the human DNA activated protein kinase (DNA-PK). DNA-PK phosphorylation substrates include RPA, p53, c-Jun, HMG1, and a variety of transcription factors, and Ku and DNA-PK have been implicated in double strand break repair and V(D)J recombination. Interestingly, cells with a

defect in DNA-PK are sensitive to cisplatin (Muller et al., 1998), and murine leukemia cells resistant

to cisplatin express higher levels of Ku (Frit et al., 1999). Biochemical experiments demonstrate that

even though the ability of Ku to recognize DNA is not affected by the presence of cisplatin adducts,

the adducts inhibit the ability of Ku to stimulate DNA-PK in a dose dependent manner (Turchi and

Henkels, 1996; Turchi et al., 1997). Moreover, the presence of a single 1,2-d(GpG), 1,2-d(ApG),

1,3-d(GpNpG), or an interstrand crosslink barely affects the affinity of Ku for DNA, but inhibits

DNA-PK activation (Turchi et al., 1997). The degree of inhibition varies depending on the specific

adduct employed. The 1,2-d(ApG) adduct causes the greatest degree of inhibition, followed by the

1,2-d(GpG) and 1,3-d(GpTpG) adducts. Adducts of *trans*-DDP are also capable of inhibiting DNA-

PK catalyzed phosphorylation of target proteins *in vitro* (Turchi et al., 1999). Interestingly, cisplatin

is used clinically in conjunction with ionizing radiation for the treatment of a variety of cancers. In

light of the aforementioned observations, Turchi *et al.* (Turchi et al., 1999) propose that cisplatin

sensitizes tumor cells to ionizing radiation via the inhibition of DNA-PK phosphorylation of target

proteins which results in a defect in double strand break repair. Accordingly, treatment with

cisplatin prior to irradiation results in a decrease in the repair of double strand breaks (Dolling et al.,

1998).

HMG Box Proteins

The HMG domain is the common structural element of the HMG1/2 family of DNA binding

proteins. These proteins can be classified into two categories. The first group contains proteins that

have multiple HMG domains and recognize DNA with little sequence specificity. They are

sometimes called structure specific HMG domain proteins, and they include HMG1, HMG2, the

upstream binding factor (UBF), and the mitochondrial transcription factor (mtTFA). The second group contains sequence specific binding proteins, including the lymphoid enhancer binding factor LEF-1 and the sex determining factor SRY. A common feature of all HMG domain proteins is their ability to bend DNA. Moreover, they have a high affinity for non canonical DNA structures such as cruciform DNA.

Several of the HMG domain proteins, as well as the purified HMG domains of these proteins, recognize cisplatin adducts and they display a selective affinity for clinically effective platinum drugs; they bind to cisplatin and Pt(en)Cl$_2$ adducts, whereas they have no affinity for adducts of *trans*-DDP or [Pt(dien)Cl]$^+$. Moreover, they bind selectively to the 1,2-d(GpG) and 1,2-d(ApG) adducts and they do not recognize the 1,3-d(GpNpG) adducts of cisplatin. HMG box proteins recognizing cisplatin damage include the human and *Drosophila* structure specific recognition protein 1 (SSRP1) (The K$_d$ of hSSRP1 for globally modified plasmid DNA is ~0.1-2 nM) (Toney et al., 1989; Donahue et al., 1990; Bruhn et al., 1992; Bruhn et al., 1993); the non histone, chromatin associated calf HMG1 (K$_d$ is ~0.3 nM for globally modified DNA) (Hughes et al., 1992; Billings et al., 1992; Turchi et al., 1996) and HMG2 (K$_d$ is ~0.2 nM for globally modified DNA) (Hughes et al., 1992; Billings et al., 1992); the rat HMG1 (K$_d$ is 370 nM for a single 1,2-d(GpG) adduct) (Pil and Lippard, 1992); the *Drosophila* homologue of HMG1, HMG-D; the *Schizosaccharomyces pombe* Cmb1 (Fleck et al., 1998); UBF (K$_d$ is 60 pM for a single 1,2-d(GpG) adduct) (Treiber et al., 1994); mtTFA (K$_d$ is 100 nM for a single 1,2-d(GpG) adduct) (Chow et al., 1994); the yeast transcription factor Ixr1 (K$_d$ is 250 nM for a single 1,2-d(GpG) adduct) (Brown et al., 1993; McA et al., 1996); the mouse testis specific tsHMG (K$_d$ is 24 nM for a single 1,2-d(GpG) adduct) (Ohndorf et al.,

1997); and the sex determining factor SRY (K_d is 120 nM for a single 1,2-d(GpG) adduct) (Trimmer et al., 1998). Direct comparisons of the binding affinities of different HMG box proteins for cisplatin adducts cannot be made because the K_d values are very sensitive to sequence context; for example the K_d of HMG1 domain A for a 1,2-d(GpG) adduct ranged from 1.6 nM to 517 nM depending on the sequence context (Dunham and Lippard, 1997).

Structural considerations

HMG box proteins distort DNA upon binding and stabilize bent and supercoiled DNA. The HMG domain from both sequence specific and structure specific HMG domain proteins has an L-shaped fold involving three α helices. The same fold is observed in the NMR solution structures of the sequence specific HMG domains of LEF-1 and SRY bound to their cognate recognition sequences (Love et al., 1995; Werner et al., 1995a; Werner et al., 1995b). The domain binds in the minor groove and causes bending and unwinding of the DNA helix, resulting in a widened minor groove and a compressed major groove. An amino acid side chain (methionine and isoleucine respectively) intercalates into the DNA duplex from the minor groove side at the site of the bend and stabilizes it. The complexes of the *S. cerevisiae* HMG non histone protein 6A in complex with DNA containing the recognition sequences of SRY and LEF1 have also been studied by NMR (Allain et al., 1999). In these complexes the DNA architecture is distorted, and Met 29 and Phe 48 intercalate between adjacent base pairs, generating two kinks in the DNA. Finally, isoleucine intercalation is observed in the structure of the HMG domain of SRY in complex with four way junction DNA (Peters et al., 1995). These results suggest that intercalation of a hydrophobic residue into the DNA helix might be an important determinant for substrate recognition by HMG domain proteins.

The crystal structure of rat HMG1 domain A in complex with DNA containing a single 1,2-d(GpG) adduct was also solved (Ohndorf et al., 1999). The DNA is bent by 61° towards the major groove, and the minor groove is widened. Moreover, the Phe 37 side chain intercalates through the minor groove into a hydrophobic notch generated by the destacking of the platinated guanines. A Phe37Ala mutation greatly reduced the affinity for the 1,2-d(GpG) adduct consistent with the hypothesis that intercalation plays a role in substrate recognition.

The structure of rat HMG1 domain A with the 1,2-d(GpG) adduct might explain the previously puzzling observation that HMG1 recognizes the interstrand crosslink formed by cisplatin (Kasparkova and Brabec, 1995). The structure of an interstrand crosslink of cisplatin reveals that the cytosines complementary to the platinated guanines are extrahelical (Huang et al., 1995; Paquet et al., 1996; Coste et al., 1999). This structure is very similar to the structure of a self-complementary oligonucleotide with two central G: T mismatches in complex with the MUG glycosylase (Barrett et al., 1998). As mentioned above glycosylases rotate bases out of the DNA helix and the crystal structure of AAG reveals that the glycosylases intercalate a hydrophobic residue in the place originally occupied by the everted base, thereby stabilizing the interaction (Lau et al., 1998). It is likely that HMG1 recognizes cisplatin interstrand crosslinks by intercalation of Phe 37 in the space originally occupied by cytosines.

Transcription Factor Hijacking Model

The human ribosomal RNA (rRNA) transcription factor UBF binds to a 1,2-d(GpG) cisplatin adduct with high affinity (K_d = 60 pM) (Treiber et al., 1994). For comparison the dissociation

constant for its cognate promoter is 18 pM (Treiber et al., 1994). The high affinity of hUBF for cisplatin adducts is attributed to the presence of multiple HMG boxes that contribute to binding in an additive way (Zhai et al., 1998). Moreover, hUBF binds specifically to DNA modified with clinically effective platinum drugs (cisplatin, Pt(en)Cl$_2$, DACH) and not to DNA modified with clinically ineffective *trans*-DDP or [Pt(dien)Cl]$^+$ (Zhai et al., 1998). Interestingly, levels of cisplatin adducts well below those found in patients treated with the drug are able to compete with the ribosomal RNA promoters for binding to hUBF (Treiber et al., 1994). In addition cisplatin modified DNA inhibits rRNA synthesis in a reconstituted system, and this inhibition can be reversed by addition of an excess of hUBF (Zhai et al., 1998). Accordingly, cisplatin causes a redistribution of hUBF in the nucleolus of human cells similar to that observed after inhibition of rRNA synthesis, whereas *trans*-DDP does not alter the localization of hUBF (Jordan and Carmo-Fonseca, 1998). Moreover, cisplatin treatment inhibited the synthesis of rRNA *in vivo* (Jordan and Carmo-Fonseca, 1998). The transcription factor hijacking model proposes that cisplatin adducts sequester hUBF away from its promoter, thereby disrupting the transcription of ribosomal genes that may be critical for cell survival. This model was tested *in vivo* using *S. cerevisiae* cells. Ixr1 is a yeast HMG box protein that inhibits transcription of cytochrome *c* oxidase subunit V by binding to the Cox5b promoter. Cisplatin treatment does not affect transcription from the promoter (McA'Nulty et al., 1996), indicating that cisplatin adducts cannot titrate Ixr1 away from the Cox5b promoter. It is noteworthy, however, that the dissociation constant for Ixr1 binding to a single 1,2-d(GpG) adduct is 250 nM; therefore, the binding affinity for cisplatin adducts might be insufficient to compete with the strong sequence specific interaction between Ixr1 and Cox5b.

Repair shielding Model

In vitro excision repair assays demonstrated that excision of the 1,2-d(GpG), but not of the

1,3-d(GpTpG) adduct, by the mammalian excinuclease present in cell extracts, is inhibited in the

presence of HMG1, mtTFA, testis specific HMG (tsHMG), or SRY (Huang et al., 1994; Zamble et

al., 1996; Trimmer et al., 1998). The repair shielding model proposes that HMG box proteins

mediate cisplatin toxicity by binding to cisplatin adducts and shielding them from repair. The

adducts persist in DNA, thereby potentiating their cytotoxicity. Consistent with this model, purified

RPA protein cannot displace HMG1 bound to cisplatin adducts (Patrick and Turchi, 1998).

Furthermore, *S. cerevisiae* strains deficient in the HMG domain protein Ixr1 are 2-6 fold more

sensitive to cisplatin and accumulate fewer cisplatin adducts than wild type strains (Brown et al.,

1993; McA'Nulty and Lippard, 1996). Moreover, the differential sensitivity to cisplatin is abolished

in an excision repair deficient background, suggesting that Ixr1 can shield cisplatin adducts from

repair *in vivo* (McA'Nulty and Lippard, 1996). In contrast *cmb1* deficient *S. pombe* cells are more

sensitive to cisplatin than wild type cells (Fleck et al., 1998). These results indicate that there is not

a unified mechanism for the role of HMG box proteins in cisplatin toxicity; some, such as Ixr1,

potentiate toxicity, whereas others, such as Cmb1 might alleviate toxicity.

Histone H1

Histone H1 binds to linker DNA in chromatin. Interestingly, linker histones and HMG1/2

proteins share a propensity to bend bound DNA and to bind to bent and branched DNA structures.

Accordingly, they compete for binding to four way junctions, with histone H1 exhibiting a higher

affinity than HMG1. Even though the exact function of HMG1 is now known, it has been suggested

that it functionally replaces histone H1 during the remodeling of chromatin that occurs during replication, transcription and repair. These results indicated that histone H1 might be able to recognize cisplatin adducts, and biochemical experiments confirm this hypothesis; histone H1 binds more strongly to cisplatin modified DNA than to *trans*-DDP modified or unmodified DNA.

TATA Binding Protein

The TATA binding protein (TBP) is a transcription factor required for initiation of transcription by all three eukaryotic RNA polymerases. Interestingly, the association of TBP with promoter sequences is slow and may be the rate limiting step in transcriptional activation. The crystal structures of yeast and *Arabidopsis Thaliana* TBP, in complex with their cognate TATA boxes, reveal that TBP bends DNA by 80° toward the major groove. TBP recognizes UV or cisplatin damaged DNA (Vichi et al., 1997; Coin et al., 1998). Moreover, consistent with the transcription factor hijacking model, the presence of UV adducts inhibits transcription *in vivo,* and this inhibition is reversed by microinjection of TBP into the cells (Vichi et al., 1997).

Y Box Binding Protein

The Y box binding protein (YB-1) is a transcription factor that binds to the inverted CCAAT element (Y box) in DNA. The Y box is located in the promoter sequences of many genes, such as PCNA, polymerase α, and multidrug resistance 1 (*mdr1*). Interestingly, cisplatin induces *mdr1* expression, and this induction can be attenuated by reducing cellular YB-1 levels (Ohga et al., 1998). Moreover, YB-1 is overexpressed in cisplatin resistant cell lines, and reduction of YB-1 expression sensitizes cells to cisplatin (Ohga et al., 1996). In contrast to HMG box proteins, YB-1 recognizes

both the 1,2- and 1,3-intrastrand crosslinks of cisplatin (Ise et al., 1999).

F. Resistance to Cisplatin

Intrinsic or acquired resistance to cisplatin is a major limitation in its use in cancer chemotherapy. Cisplatin resistant cell lines, isolated after prolonged exposure to low doses of the drug have been valuable in elucidating the mechanisms underlying cisplatin resistance. Postulative mechanisms operating at the cellular levels include reduction in cellular accumulation, increased inactivation by sulfur containing molecules, altered expression of regulatory genes, increased repair of the adducts, and increased adduct tolerance. It is important to note that pharmacological agents that modulate any of the above parameters only partially restore sensitivity to cisplatin, and that different studies identified different determinants of cisplatin resistance, indicating that resistance is multifactorial and underscoring the importance of studies with well defined systems, such as the use of isogenic cell lines, in order to evaluate each parameter. Moreover, the extent to which data generated from the study of cell lines that have acquired resistance after extensive passaging in the present of the drug, can be extrapolated to human tumors is uncertain. This point is underscored by the observation that cells selected for resistance *in vitro* acquire higher levels of resistance than those observed in tumors of patients that are refractory to cisplatin based chemotherapy.

Drug Uptake/Efflux as a Determinant of Cellular Sensitivity

Reduced intracellular accumulation of cisplatin, which may arise because of decreased uptake or increased efflux, is frequently observed in cisplatin resistant cell lines (Andrews and Howell, 1990; Parker et al., 1991; Dempke et al., 1992). To date the exact mechanism by which cisplatin is

taken up by the cells is not fully understood (Gately and Howell, 1993; Akiyama et al., 1999). The

rate limiting factor for cisplatin uptake is its concentration and uptake is not inhibited by structural

analogues and uptake is not saturable, suggesting that cisplatin enters the cells by passive diffusion.

In contrast a variety of pharmacological agents that do not alter the permeability of the membrane

inhibit cisplatin uptake. The sodium-potassium ATPase inhibitor ouabain inhibits uptake, and

cisplatin accumulation is potassium dependent, even though cisplatin is not transported into the cells

through the sodium-potassium pump, indicating that accumulation is dependent on cell membrane

potential. Moreover, a number of aldehydes inhibit uptake, presumably by forming Schiff bases with

membrane proteins. These results are in favor of a carrier mediated transport system for cisplatin.

Inactivation of Cisplatin by Sulfur Containing Molecules

Resistance to cisplatin because of increased inactivation by intracellular proteins has also

been reported (for a review see (Perez, 1998; Akiyama et al., 1999)). The metabolic synthesis of

glutathione (γ-glutamylcysteinylglysine, GSH) involves peptide bond formation between cysteine

and glutamic acid (rate limiting step catalyzed by γ-glutamylcysteine synthetase), followed by

peptide bond formation with glycine. GSH levels can be reduced with the use of buthione-S-

sulfoxime (BSO), which is an inhibitor of γ-glutamylcysteine synthetase. Cisplatin can be covalently

linked to GSH after nucleophilic attack of the glutathione thiolate anion (Ishikawa and Ali-Osman,

1993), and this complex can be transported out of the cell by an ATP-dependent pump (Ishikawa and

Ali-Osman, 1993). Conjugation with GSH inhibits the conversion of monoadducts to crosslinks,

thereby reducing the cytotoxic potential of the adducts. In addition GSH might protect cells by

maintaining the dNTP pool size needed for DNA repair and by maintaining functional repair

enzymes such as polymerase α (Lai et al., 1989). Interestingly, elevated GSH levels have been found in some cisplatin resistant cell lines and depletion of GSH by BSO increased cisplatin sensitivity in some cell lines but not in others (Hamilton et al., 1985; Andrews et al., 1985; Hromas et al., 1987; Hospers et al., 1988; Mistry et al., 1991; Godwin et al., 1992).

Metallothioneins are a family of cysteine rich proteins involved in Zn^{2+} homeostasis and in the detoxification of heavy metals such as cadmium. Metallothioneins bind to cisplatin in a ratio of 1:10 and may affect sensitivity to the drug. Metallothionein deficient mouse fibroblasts are more sensitive than wild type fibroblasts (Kondo et al., 1995); overexpression of metallothionein can sometimes cause resistance to cisplatin (Kelley et al., 1988); and conversely cell lines that have acquired resistance to cisplatin overexpress metallothionein (Kasahara et al., 1991). Moreover, cadmium resistant cell lines overexpress metallothionein and are cross resistant to cisplatin (Bakka et al., 1981; Andrews et al., 1987). In contrast cisplatin resistant cells are slightly cross resistant to cadmium or show no resistance. In addition analysis of the amount of metallothionein in samples from ovarian tumors before and after chemotherapy reveal that metallothionein content is not a major determinant of sensitivity to cisplatin based chemotherapy (Murphy et al., 1991).

Regulatory Proteins

Alterations in the expression of oncogenes (such as *fos, ras, jun, v-abl, myc,* and *Her/neu*) and tumor suppressor genes (such as p53) have also been implicated in the cellular resistance to cisplatin. Since a change in the expression of these genes can have pleiotropic effects on cellular homeostasis, the mechanism underlying resistance is not entirely understood. Overexpression of *ras,*

fos, c-jun, and *myc* increases resistance to cisplatin, and downregulation of *c-jun* sensitizes cells to cisplatin (Scanlon et al., 1991). *c-fos* modulates the expression of genes that have the AP-1 (fos/jun complex) binding domain, such as *c-myc*, metallothionein, and DNA polymerase β. Metallothionein and DNA polymerase β expression can also be modulated by *H-ras*. The tumor suppressor gene p53 is involved in cell cycle control, DNA repair and apoptosis. Specifically, p53 activates cell cycle arrest at G1 or G2/M phases in response to DNA damage, thereby extending the time available to repair the damage. p53 can also play a direct role in repairing of the damage or it can initiate an apoptotic response to DNA damage. Consequently, a defect in p53 can have pleiotropic effects in the cellular sensitivity to cisplatin. Lymphoma cells, ovarian cancer cells, and lung cancer cells mutated in p53 are more resistant to cisplatin than wild type cells (Fan et al., 1994; Eliopoulos et al., 1995; Perego et al., 1996), presumably because the p53 dependent apoptotic response is inactivated. In contrast p53$^{-/-}$ mouse fibroblasts are more sensitive to cisplatin than wild type cells (Hawkins et al., 1996), and inactivation of p53 in human foreskin cells, breast cancer cells, and colon cancer cells sensitizes them to cisplatin (Fan et al., 1995; Hawkins et al., 1996), presumably because these cells are not as susceptible to apoptosis and p53 can facilitate repair and extend the time available for repair.

DNA Repair Status and Cellular Sensitivity to Cisplatin

Cell lines selected for resistance to cisplatin after prolonged culture in the presence of cisplatin have significantly higher levels of repair than the corresponding parental cell lines (Masuda et al., 1988; Parker et al., 1991; Dempke et al., 1992; Nishikawa et al., 1992; Johnson et al., 1994), indicating that DNA repair is an important determinant of cisplatin resistance. Moreover, differential

capacity to repair cisplatin adducts is postulated to be responsible for the variability in clinical response to cisplatin based chemotherapy (Jones et al., 1994). Interestingly, protein extracts from ovarian tumor biopsies vary in their abilities to repair cisplatin adducts by up to 10 fold, and this variability in repair capacity is an intrinsic property of the tumor and does not correlate to the repair capacity of non tumor cells from the same individual (Jones et al., 1994). In general even though ovarian cancer patients have initially high response rates to chemotherapy, frequently resulting in complete remission, they often relapse and their tumors become refractory to subsequent chemotherapy. Interestingly, the sensitivity of ovarian carcinoma xenographs established from the same patients at different stages of the disease reflected the responsiveness of the patient to chemotherapy (Masazza et al., 1991), underscoring the usefulness of cell culture studies. Moreover, a cell line established from the tumor of an ovarian cancer patient that was not responding to chemotherapy had a three fold higher repair synthesis activity than the cell line established from the tumor of the patient prior to the onset of resistance (Lai et al., 1988). A similar increase in repair is observed in cells of an oligodendroglioma obtained after the onset of resistance as compared to tumor cells obtained prior to therapy. The increase in repair is correlated with higher expression levels of DNA polymerase β (Ali-Osman et al., 1994).

Cell lines established from testicular tumors appear to be more sensitive to cisplatin than other cultured cell lines, reflecting the clinical responsiveness of these tumors. For example, testicular tumor cell lines are, on average, 4 fold more sensitive to cisplatin than bladder tumor cell lines (Bedford et al., 1988). Moreover, following 18 hrs of incubation after cisplatin treatment, five out of six testicular teratoma cell lines (including the SuSa cell line) had adduct levels similar to

those observed immediately after treatment, indicating that these cell lines have a significantly reduced capacity to remove cisplatin adducts (Bedford et al., 1988; Hill et al., 1994). The sixth repair proficient cell line (833K) was established from a patient that had received platinum based chemotherapy and had higher polymerase β levels (Hill et al., 1994). It is noteworthy that similar experiments from a different group indicate that the 833K cell line is repair deficient, and the SuSa cell line is repair proficient (Koberle et al., 1997). Accordingly, SuSa cells and repair proficient cells were capable of reactivating adenovirus DNA modified with cisplatin to a similar extent (Maynard et al., 1989). These differences may be attributed to the different concentrations of cisplatin used in the first two experiments (17 μM versus 50 μM) and/or the different time of analysis (18 hrs versus 48 or 72 hrs). Consistent with the hypothesis that testicular tumors have low capacity for cisplatin adduct repair, extracts from three testicular tumor cell lines (including 833K) support low levels of excision of a 1,3-d(GpTpG) cisplatin adduct (Koberle et al., 1999). Western analysis indicates that even though the testicular tumors have high levels of most repair proteins, such as RPA, XPG, and XPC, they have low levels of XPA and ERCC1-XPF (Koberle et al., 1999). Moreover, addition of these proteins in the reactions stimulates repair (Koberle et al., 1999), suggesting that testicular tumors are deficient in nucleotide excision repair and are therefore less able to tolerate cisplatin induced damage.

Even though small cell lung cancer (SCLC) has a very aggressive clinical course with median survival after diagnosis of only two to four months, it is more responsive to chemotherapy than non-small cell lung cancer (N-SCLC). Accordingly, primary and established N-SCLC cell lines are more resistant to cisplatin, and they have a higher overall capacity to repair cisplatin adducts, as measured

by their ability to reactivate cisplatin damaged plasmid DNA, than SCLC cell lines (Shellard et al., 1993; Schmidt and Chaney, 1993; Zeng-Rong et al., 1995).

Adduct Tolerance

A study of a panel of human ovarian cancer cell lines derived from patients who were or were not treated with cisplatin based chemotherapy revealed that adduct level tolerance correlated well with sensitivity to cisplatin (Johnson et al., 1997). Moreover, several reports have demonstrated that cisplatin resistant cells have a higher capacity for adduct tolerance than the corresponding cisplatin sensitive parental cell lines (Parker et al., 1991; Mamenta et al., 1994).

Replication Bypass

The enhanced tolerance of cisplatin adducts has been correlated with the increased ability to replicate past cisplatin adducts. A three to four fold increase in replication bypass of $Pt(en)Cl_2$ adducts is observed in a murine leukemia resistant cell line (Gibbons et al., 1991), and the A2780/DDP ovarian carcinoma cell line has a 2.3 fold increased ability to replicate past cisplatin adducts than the parental A2780 cisplatin sensitive cell line (Mamenta et al., 1994). Moreover, the C13* ovarian cancer cell line demonstrates a 4.5 fold increase in replicative bypass ability over the corresponding 2008 cisplatin sensitive cell line (Mamenta et al., 1994). Even though the DACH analogue of cisplatin causes a greater inhibition of DNA chain elongation than cisplatin in the parental 2008 cell line, the level of enhanced bypass of DACH DNA adducts is two fold lower than that of cisplatin adducts in the resistant cell line (Mamenta et al., 1994). Interestingly, mismatch repair deficient cells are resistant to cisplatin but not to oxaliplatin (which forms the same types of

adducts as DACH) (Fink et al., 1996; Drummond et al., 1996; Aebi et al., 1996; Fink et al., 1997), indicating that mismatch repair deficient cells are better able to tolerate cisplatin adducts and suggesting that mismatch repair is involved in replicative bypass of cisplatin adducts. Consistent with this hypothesis, cells with *hMLH1* or *hMSH6*, but not *hMSH3* deficiencies have a 2.5-6 fold increased ability to replicate past cisplatin adducts, whereas they do not display a significant difference in the bypass of oxaliplatin adducts (Vaisman et al., 1998).

Replication bypass of cisplatin adducts has also been examined *in vitro*. The ability of bacteriophage T7 and bacteriophage T4 DNA polymerases, Taq polymerase, *E. coli* polymerase I, and *E. coli* polymerase III holoenzyme to replicate past cisplatin adducts has been examined in an *in vitro* primer elongation assay (Comess et al., 1992). These polymerases are able to synthesize past cisplatin adducts with different efficiencies. T4 DNA polymerase is strongly inhibited by all the cisplatin intrastrand crosslinks, demonstrating only 2% translesion synthesis. The 1,2-d(GpG) adduct inhibits replication very efficiently (2-6% translesion synthesis) by all DNA polymerases. Interestingly, the 1,3-d(GpTpG) adduct is more inhibitory than the 1,2-d(ApG) adduct when Taq polymerase or polymerase III is used (5% versus 9% and 4% versus 9% translesion synthesis, respectively), whereas T7 DNA polymerase or *E. coli* polymerase I are more inhibited by the 1,2-d(ApG) adduct than the 1,3-d(GpTpG) adduct (6% versus 25% and 14% versus 27% translesion synthesis, respectively).

The ability of eukaryotic DNA polymerases to replicate past cisplatin adducts has also been determined. Human polymerase β, γ, η (the product of the XP-V gene), yeast polymerase ζ

(Rev3/Rev7 complex), and human immunodeficiency virus type I reverse transcriptase can bypass a 1,2-d(GpG) cisplatin adduct with an efficiency of 5-15% relative to unmodified DNA (Hoffmann et al., 1995; Hoffmann et al., 1996; Hoffmann et al., 1996; Hoffmann et al., 1997; Vaisman et al., 1999; Vaisman and Chaney, 2000; Vaisman et al., 2000). Moreover, bypass of the 1,2-d(GpG) adduct by polymerase β is more efficient when the adduct is placed in a fork like substrate than when situated in single stranded DNA (Hoffmann et al., 1995). In contrast polymerase α, δ and ε are blocked by this adduct and addition of PCNA or RPA to the reactions does not alter the ability of polymerase δ or ε to replicate past the adduct (Hoffmann et al., 1995). Interestingly, yeast polymerase ζ and human polymerases β, γ, and η bypass oxaliplatin 1,2-d(GpG) adducts more efficiently than cisplatin 1,2-d(GpG) adducts (Vaisman et al., 1999; Vaisman et al., 2000). The hypothesis that mismatch repair proteins modulate translesion synthesis past cisplatin adducts more efficiently than past oxaliplatin adducts *in vivo* could be very easily tested in this system by monitoring translesion synthesis in the presence of hMSH2 or other mismatch repair proteins. Interestingly, in this system, HMG1 blocks bypass of cisplatin adducts 2.5 fold more effectively than translesion synthesis past oxaliplatin adducts (Hoffmann et al., 1997; Vaisman et al., 1999).

The importance of translesion bypass by polymerase β has been determined *in vivo*. Downregulation of polymerase β by antisense RNA sensitizes mouse fibroblasts to cisplatin (Horton et al., 1995). Conversely, increased expression of polymerase β in Chinese hamster ovary cells confers resistance to cisplatin and increases the mutagenic potency of cisplatin (Canitrot et al., 1998). Murine leukemia and colorectal cancer cells that have acquired resistance to cisplatin *in vitro* have higher levels of polymerase β expression (Kraker and Moore, 1988; Yang et al., 1992). Moreover,

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cells of a malignant oligodendroglioma obtained from a patient before chemotherapy expressed

lower levels of polymerase β when compared to cells from the same tumor acquired after the patient

failed platinum based chemotherapy (Ali-Osman et al., 1994). Interestingly, the resistant cells also

demonstrated an increased ability to reactivate plasmid DNA damaged with cisplatin (Ali-Osman

et al., 1994). Finally, polymerase β expression observed in drug resistant tumors of two patients is

increased compared to normal colon tissue (Kashani-Sabet et al., 1990).

Fidelity of Translesion Synthesis

The mutagenic consequence of replication bypass of a 1,2-d(GpG) adduct by polymerase β

was determined *in vitro*. In 11% of the products a -1 deletion of the cytosine located immediately

5' to the adduct is observed, 25% of the products have multiple mutations, and 58% of the products

do not contain any mutations (Hoffmann et al., 1996). Subsequent studies revealed that polymerase

β misincorporates thymine opposite the 3' platinated guanine at a frequency that is 15-25 fold higher

than the frequency of thymine misincorporation opposite undamaged guanine in the same sequence

(Vaisman and Chaney, 2000). Polymerase β can also misincorporate thymine opposite the 5'

platinated guanine, albeit at a much lower frequency (Vaisman and Chaney, 2000). Interestingly,

the fidelity of polymerase η past the GG sequence was unaffected by the presence of the platinum

adduct in that sequence (Vaisman et al., 2000).

Polymerase β can replicate past a 1,2-d(GpG) adduct located on codon 13 of the human *H-*

ras oncogene *in vitro* (Hoffmann et al., 1995), and the mutagenic potential of a 1,2-d(GpG) adduct

located in the same sequence in single stranded vector was determined in simian COS-7 cells

(Pillaire et al., 1994). The overall mutation frequency is 21% *in vivo*, compared to 42% *in vitro*. Furthermore, the most frequent modifications *in vivo* are base substitutions, most of which occur at one of the platinated guanines. The difference in these results may be attributed to the presence of accessory factors and repair systems *in vivo*. Moreover, other DNA polymerases can replicate past the cisplatin adducts, and they might be responsible for the substitutions observed *in vivo*.

Apoptosis

In addition to replication bypass, adduct tolerance could also be attained by inhibition of apoptosis. For example, cells that express high levels of an apoptosis inhibitor, such as Bcl-2, or low levels of an apoptosis promoter, such as Bax, would require higher levels of damage before they initiate apoptosis. Accordingly, cells overexpressing Bcl-2 and cells with reduced levels of Bax are more resistant to cisplatin (Dole et al., 1994; Eliopoulos et al., 1995; Miyashita et al., 1995; Perego et al., 1996).

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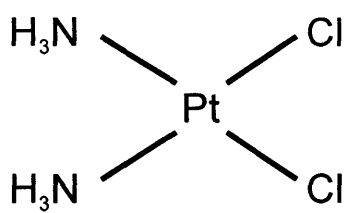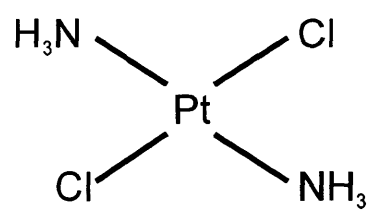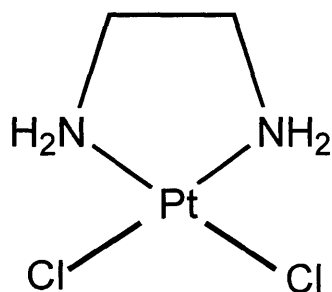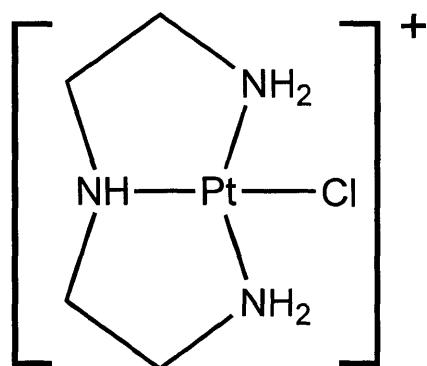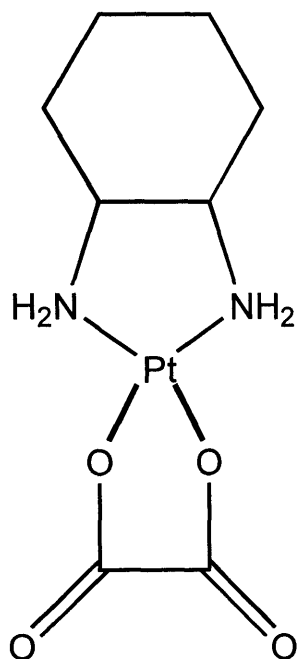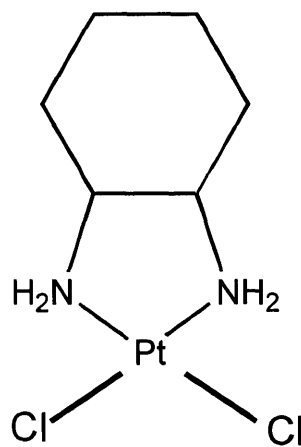
Figure 1.1: Chemical structures of platinum drugs.

Chapter 2

Cisplatin Adducts Inhibit 1,N^6-Ethenoadenine Repair by Interacting with the Human 3-Methyladenine DNA Glycosylase

A. Introduction

DNA glycosylases are an extensive family of enzymes responsible for the first step of base excision repair. Specifically, they recognize certain abnormal DNA bases and catalyze the hydrolysis of the N-glycosydic bond between the modified base and the sugar-phosphate backbone (Krokan et al., 1997). *Escherichia coli* possesses two glycosylases that are involved in the repair of 3-methyladenine (3-mA) in DNA. The 3-methyladenine DNA glycosylase I, or Tag, encoded by the *tag* gene, is constitutively expressed and removes 3-mA and, to a lesser degree, 3-methylguanine (3-mG) from DNA (Thomas et al., 1982; Bjelland et al., 1993; Bjelland and Seeberg, 1996). The 3-methyladenine DNA glycosylase II, or AlkA, is the product of the *alkA* gene and is induced during the adaptive response to alkylation damage. AlkA catalyzes the excision of a variety of alkylated bases, including 3-mA, 7-methylguanine (7-mG), 7,8-dihydro-8-oxoguanine (8-oxoG), hypoxanthine (Hx), 1,N^6-ethenoadenine (εA), oxidized products of thymine, and adducts formed by nitrogen mustards (Thomas et al., 1982; Carter et al., 1988; Habraken and Ludlum, 1989; Habraken et al., 1991a; Habraken et al., 1991b; Matijasevic et al., 1992; Matijasevic et al., 1993; Bjelland et al., 1994; Saparbaev and Laval, 1994; Seeberg et al., 1995; Bjelland and Seeberg, 1996; Matijasevic et al., 1996; Mattes et al., 1996; Berdal et al., 1998). 3-Methyladenine DNA glycosylases identified in eukaryotic cells include the mouse (Aag) and the human (AAG, also known as MPG or ANPG) enzymes, which can remove 3-mA, 3-mG, and 7-mG from DNA (Engelward et al., 1993; Samson et al., 1991; Chakravarti et al., 1991; O'Connor and Laval, 1991). Additionally, mammalian 3-methyladenine DNA glycosylases remove Hx, possibly 8-oxoG, and bases with etheno substitutions (Singer et al., 1992; Matijasevic et al., 1993; Dosanjh et al., 1994a; Dosanjh et al., 1994b; Saparbaev and Laval, 1994; Saparbaev et al., 1995; Mattes et al., 1996; Berdal et al., 1998; Wyatt et al., 1999).

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In particular, AAG is very efficient in releasing εA from DNA (Singer et al., 1992; Dosanjh et al., 1994b; Saparbaev et al., 1995; Hang et al., 1997; Roy et al., 1998; Hang et al., 1998) and it has been suggested that this naturally occurring DNA modification might be the principal physiological substrate of this repair enzyme.

The structure of AAG in a complex with DNA containing a modified apurinic/apyrimidinic (AP) site lends insight into the mechanism by which AAG recognizes this diverse group of substrates (Lau et al., 1998). The DNA is bent by 22° and the AP site analogue is rotated out of the DNA helix and into the active site of the enzyme. The active site is lined with aromatic residues that provide stacking interactions with the extrahelical residue, and a tyrosine side chain intercalates into DNA in the space originally occupied by the ejected residue further stabilizing the interaction.

Interestingly, the mouse Aag protein appears to play a role in protecting cells from killing by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and mitomycin C (MMC), both of which are used in cancer chemotherapy (Matijasevic et al., 1993; Engelward et al., 1996; Allan et al., 1998). Furthermore, the human and rat 3-methyladenine DNA glycosylases excise adducts formed by nitrogen mustards (Mattes et al., 1996) and *Saccharomyces cerevisiae* cells deficient in 3-methyladenine DNA glycosylase are more sensitive to nitrogen mustards than the corresponding wild type cells (McHugh et al., 1999). Accordingly, significant effort has been put into elucidating the role of AAG as a protective agent in cells treated with a cancer chemotherapeutic agent.

Cisplatin is an excellent example of a successful cancer chemotherapeutic agent (Zamble and

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εA
5′-CCTCTCCTTGεATCTTCTCCTCTCC-3′
3′-GGAGAGGAAC TAGAAGAGGAGAGG-5′

1,2-d(ApG) Pt
5′-CCTCTCCTT**AG**TCTTCTCCTCTCC-3′
3′-GGAGAGGAATCAGAAGAGGAGAGG-5′

1,2-d(GpG) Pt
5′-CCTCTCCTT**GG**TCTTCTCCTCTCC-3′
3′-GGAGAGGAACCAGAAGAGGAGAGG-5′

1,3-d(GpTpG) Pt
5′-CCTCTCCTT**GTG**TCTTCTCCTCTCC-3′
3′-GGAGAGGAACACAGAAGAGGAGAGG-5′

Figure 2.1: Sequences of the DNA duplexes used in the binding and glycosylase experiments. The pyrimidine rich strand is designated as the top strand, and the complementary strand is designated as the bottom strand. The bases involved in adduct formation are located in the top strand and are indicated in bold.
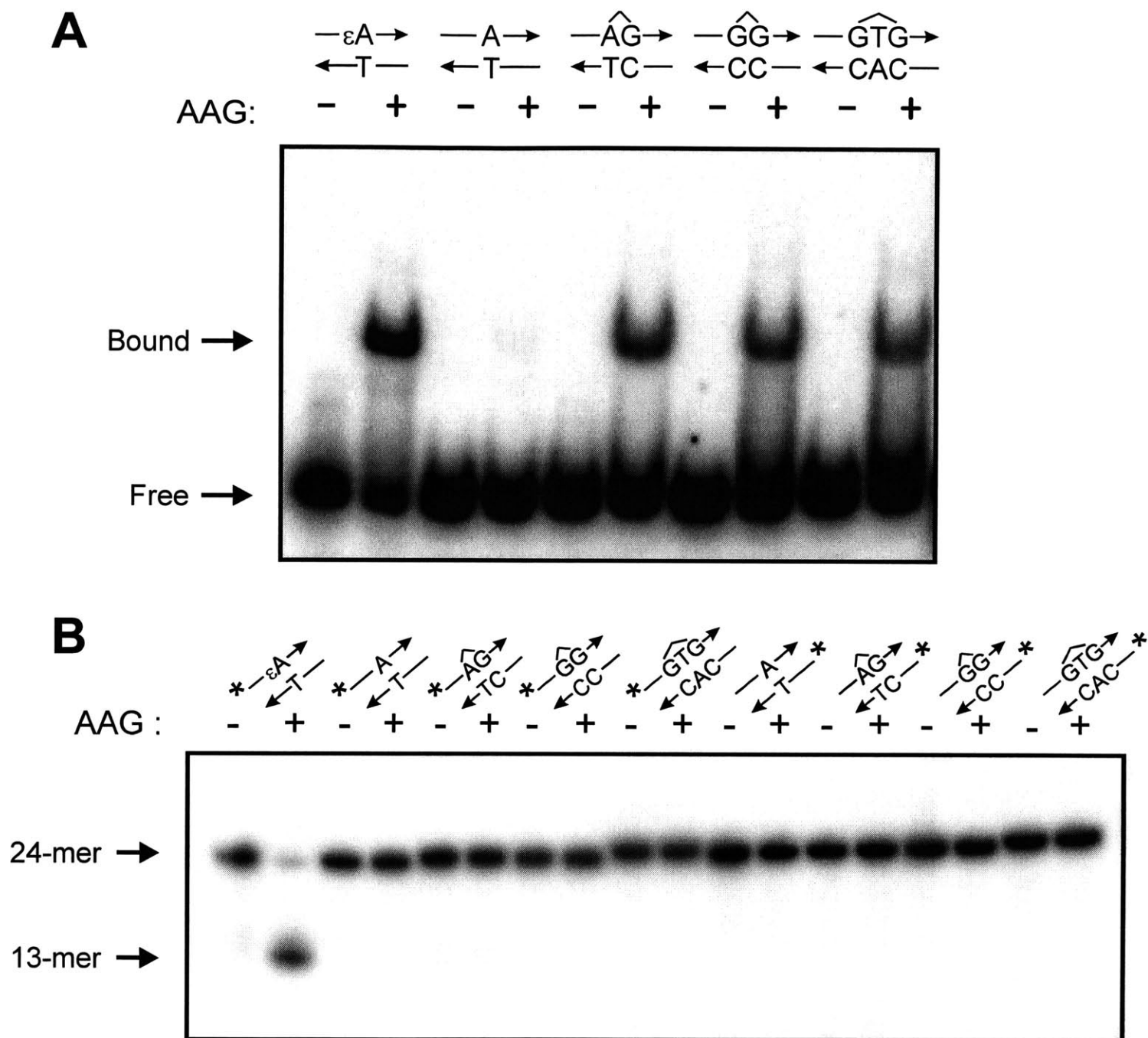
Figure 2.2: AAG recognizes the cisplatin intrastrand crosslinks. **(A)** Gel mobility shift assay of human AAG with various oligonucleotide duplexes. The ^{32}P-labeled oligonucleotides containing a single εA (lanes 1 and 2), 1,2-d(ApG) (lanes 5 and 6), 1,2-d(GpG) (lanes 7 and 8), or 1,3-d(GpTpG) (lanes 9 and 10) were incubated in the presence (+) or absence (-) of 360 nM human AAG. Unmodified DNA (lanes 3 and 4) was used as a control. **(B)** Enzymatic processing of εA or cisplatin adducts by human AAG. 1 μM human AAG was incubated with εA oligonucleotide (lanes 1 and 2), unmodified oligonucleotide (lanes 3, 4, 11, and 12), 1,2-d(ApG) oligonucleotide (lanes 5, 6, 13, and 14), 1,2-d(GpG) oligonucleotide (lanes 7, 8, 15, and 16), or 1,3-d(GpTpG) oligonucleotide (lanes 9, 10, 17, and 18), ^{32}P-labeled in the top (lanes 1-10) or in the bottom strand (lanes 11-18).
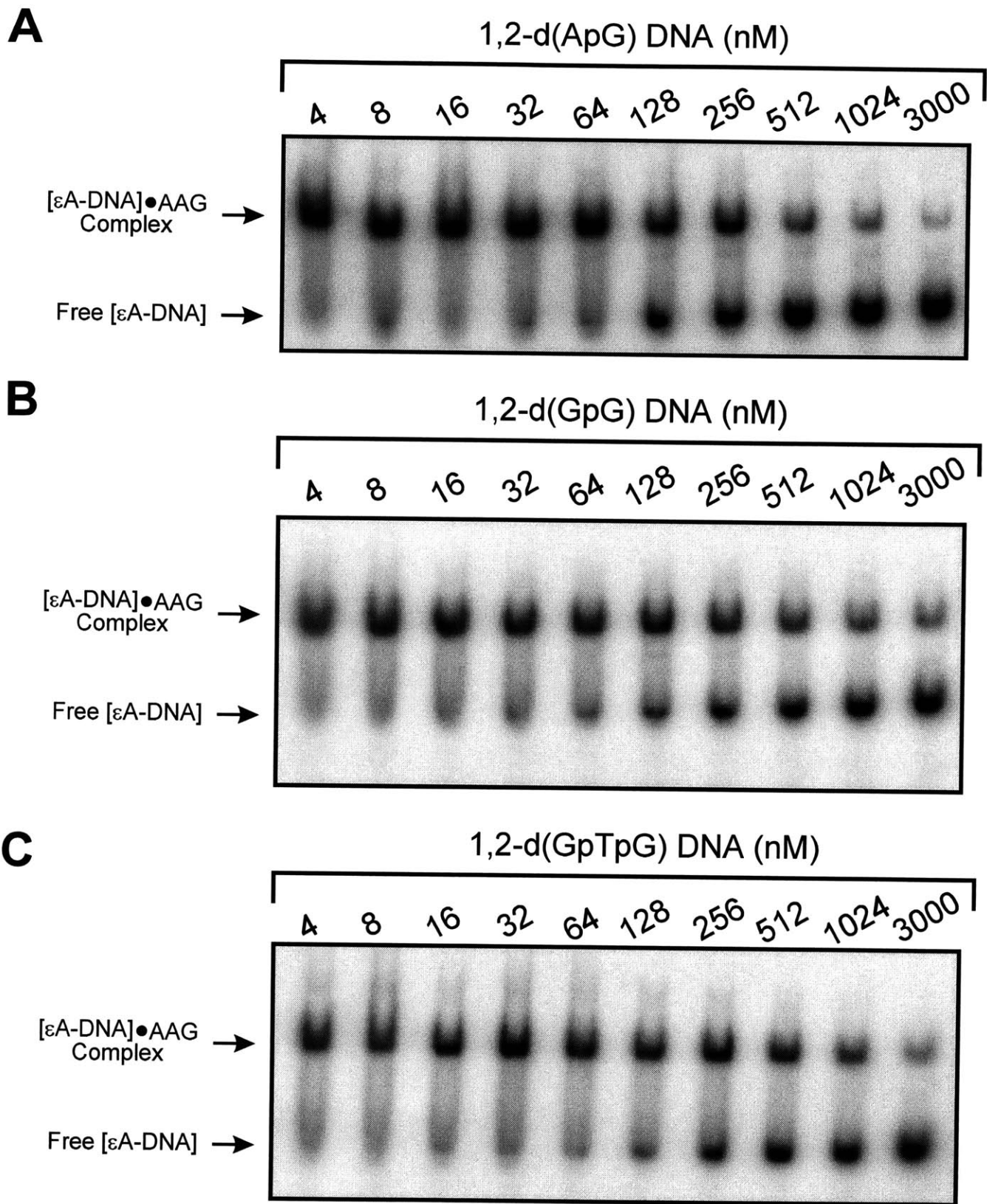
Figure 2.3: Cisplatin adducts inhibit binding of AAG to εA oligonucleotides. Unlabeled duplex DNA (with or without adducts) was used to compete the binding of AAG to ^{32}P-labeled εA duplex DNA. **(A)** Unlabeled 1,2-d(ApG) oligonucleotide was titrated into binding reaction mixtures containing 61 nM of purified human AAG protein and 0.2 nM ^{32}P-labeled εA duplex DNA. **(B)** Unlabeled 1,2-d(GpG) oligonucleotide was titrated into binding reaction mixtures containing 61 nM of purified human AAG protein and 0.2 nM ^{32}P-labeled εA duplex DNA. **(C)** Unlabeled 1,3-d(GpTpG) oligonucleotide was titrated into binding reaction mixtures containing 61 nM of purified human AAG protein and 0.2 nM ^{32}P-labeled εA duplex DNA.
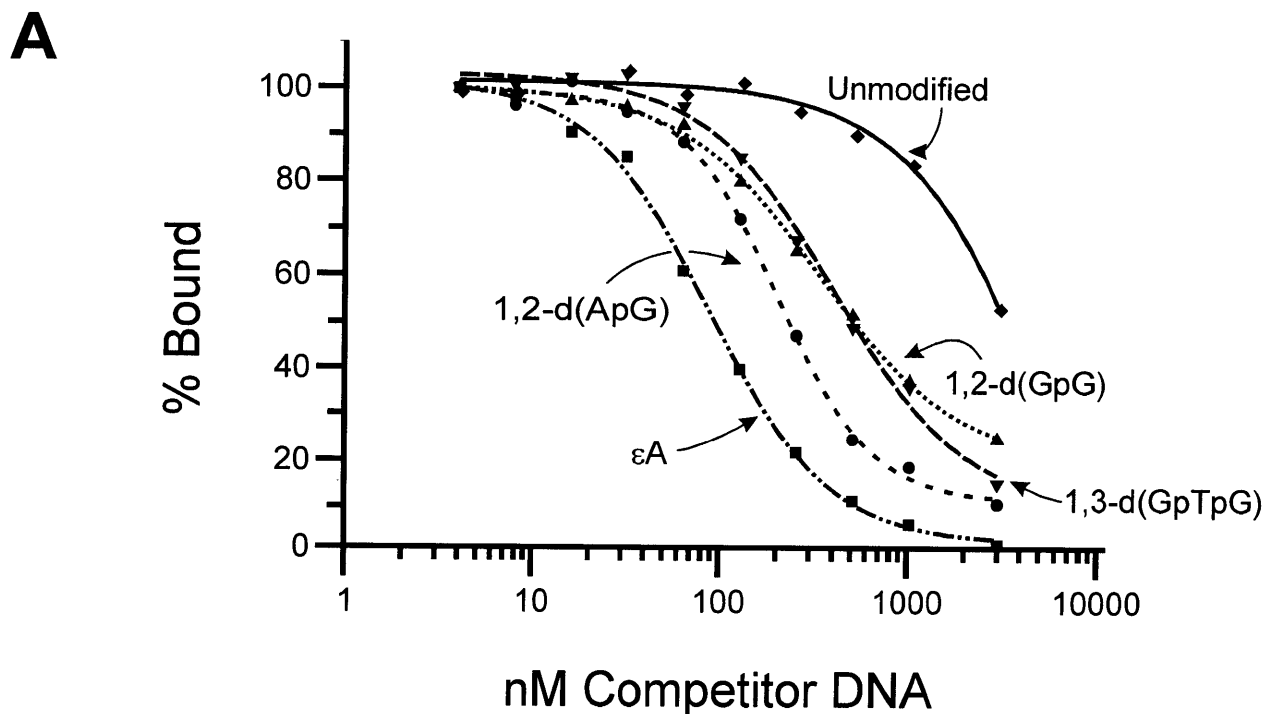
Figure 2.4: Specificity of AAG binding to cisplatin adducts. **(A)** The fraction of bound ^{32}P-labeled εA duplex DNA for each concentration of competitor DNA was quantitated by PhosphorImager analysis and is represented as a fraction of the concentration of competitor DNA present in the binding reactions. One hundred percent is the maximum amount of specific complex of AAG with the ^{32}P-labeled εA oligonucleotide, which was 65% of the total ^{32}P-labeled εA oligonucleotide; i.e., 65% of the ^{32}P-labeled εA oligonucleotide was in complex with AAG when 4 nM 1,2-d(ApG) oligonucleotide competitor DNA were present in the binding reaction. **(B)** DNA binding affinities of the human AAG protein for each competitor DNA were determined using equation 1.
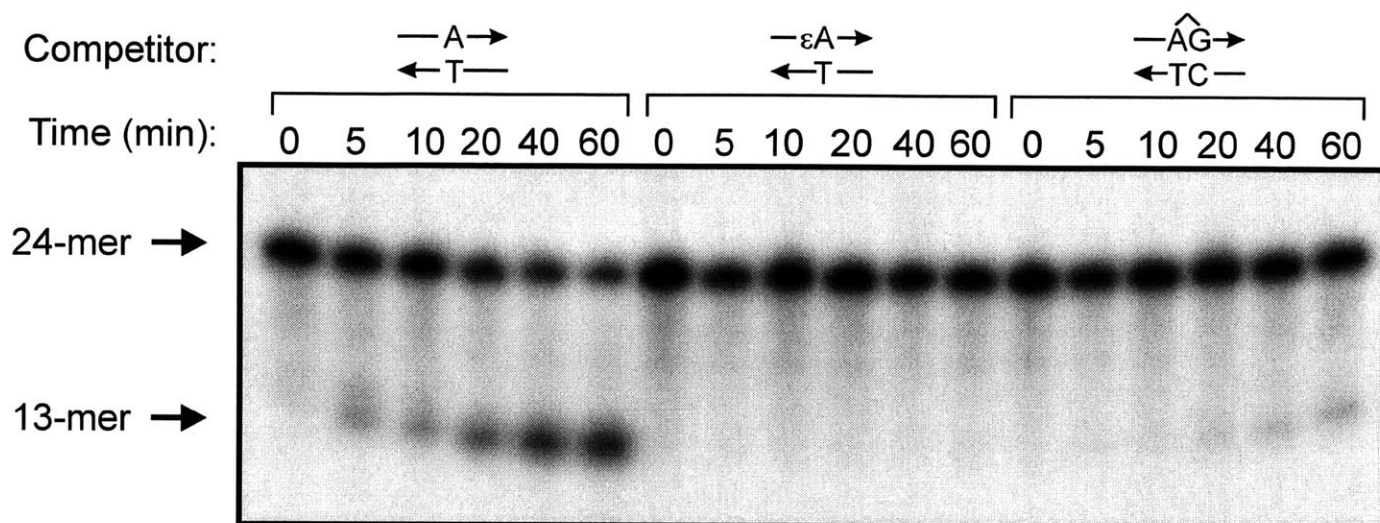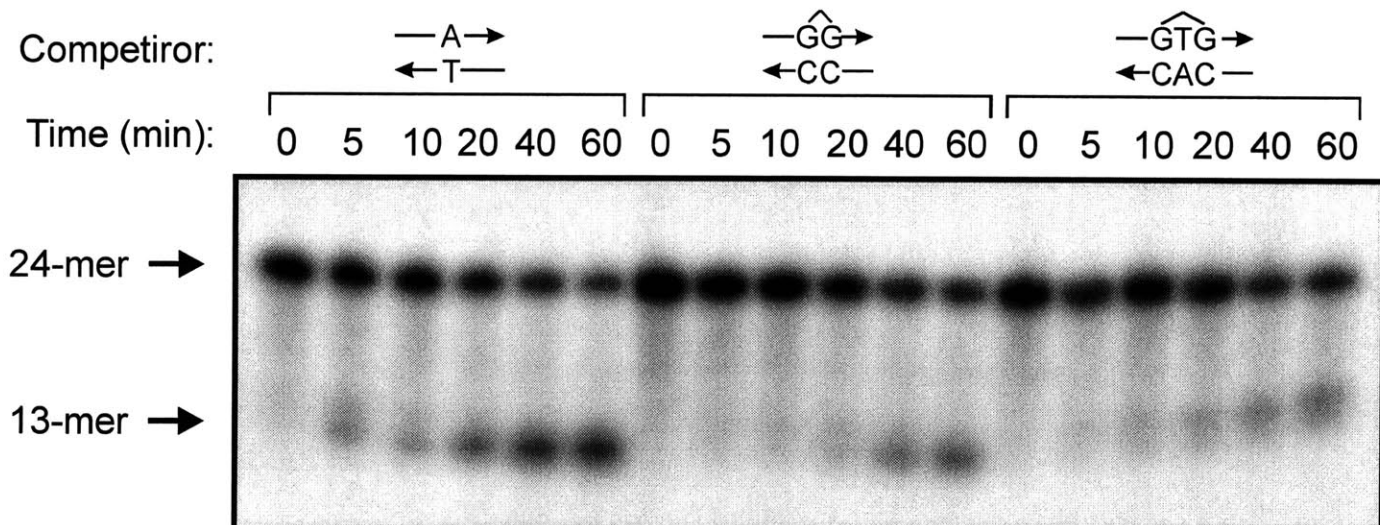
Figure 2.5: Inhibition of εA repair by cisplatin adducts. **(A and B)** ^{32}P-labeled εA oligonucleotide was incubated with human AAG in the presence of 2,500 nM unlabeled competitor DNA and removal of εA from the duplex DNA was measured over time. **(A)** Lanes 1-6: unmodified DNA; lanes 7-12: εA; lanes 13-18: 1,2-d(ApG). **(B)** Lanes 1-6: unmodified DNA; lanes 7-12: 1,2-d(GpG); lanes 13-18: 1,3-d(GpTpG).

Figure 2.6: Inhibition of εA repair by cisplatin adducts. **(A and B)** ³²P-labeled εA oligonucleotide was incubated with human AAG in the presence of 80 to 2,560 nM unlabeled competitor DNA and removal of εA from the duplex DNA after 15 min. **(A)** Lane 1: no competitor DNA; lanes 2-7: unmodified DNA; lanes 8-13: εA; lanes 14-19: 1,2-d(ApG). **(B)** Lane 1: no competitor DNA; lanes 2-7: 1,2-d(GpG); lanes 8-13: 1,3-d(GpTpG).
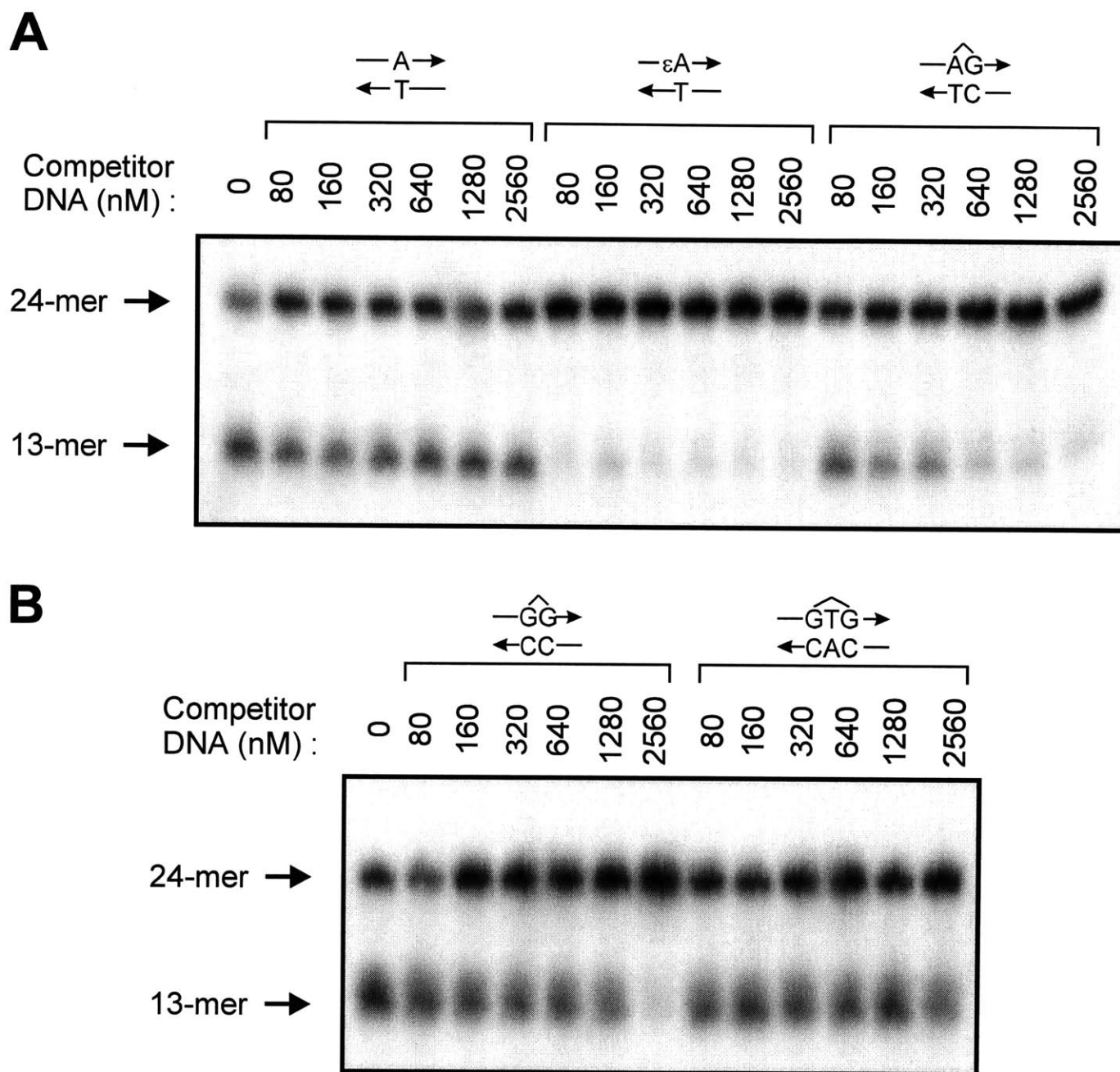
Lippard, 1995; Mello et al., 1998; Jamieson and Lippard, 1999). It is most effective in the treatment

of testicular tumors where cisplatin based combination chemotherapy can afford cure rates of over

90% (Feuer et al., 1993). Cisplatin is also used in the treatment of ovarian, bladder, head and neck,

and non-small cell lung cancer (Jamieson and Lippard, 1999), although survival in these cases is

usually limited due to acquired resistance to the drug. Even though the exact mechanism of action

of cisplatin is not understood, it is believed that the cytotoxic effects of the drug arise from processes

that are triggered by reaction with DNA (Bruhn et al., 1990). The structural alterations induced in

DNA as a result of the interaction with cisplatin have been extensively studied. DNA treated *in vitro*

with cisplatin, as well as DNA isolated from cells of patients that have been treated with cisplatin,

contains approximately 65% 1,2-d(GpG), 25% d(ApG), and 5-10% 1,3-d(GpNpG) intrastrand

crosslinks, and a small percentage of interstrand crosslinks and monofunctional adducts (Eastman,

1983; Eastman, 1985; Fichtinger-Schepman et al., 1985; Fichtinger-Schepman et al., 1987).

Each cisplatin adduct distorts the DNA architecture in a unique manner, and it has been

suggested that recognition of these altered DNA structures by cellular proteins plays an important

role in the mechanism of toxicity of cisplatin (Zamble and Lippard, 1995; Mello et al., 1998;

Jamieson and Lippard, 1999). Of particular interest has been a family of proteins with a high

mobility group domain (HMG box), which recognizes bent DNA structures, four way junctions, and

cruciform DNA (Lilley, 1992; Landsman and Bustin, 1993; Grosschedl et al., 1994; Baxevanis and

Landsman, 1995). The HMG box proteins have also been shown to recognize specifically the

adducts of clinically effective platinum drugs, and it has been suggested that the bending and

unwinding induced by cisplatin adducts (Bellon and Lippard, 1990; Bellon et al., 1991) provide a

structural signal for HMG box protein recognition (Donahue et al., 1990; Bruhn et al., 1992).

The observation that DNA is bent when in complex with AAG and when it is coordinated by cisplatin (Bellon et al., 1991; Lau et al., 1998; Ohndorf et al., 1999) led us to hypothesize that cisplatin adducts could be recognized by human AAG. Moreover, the intercalation of an HMG box protein amino acid residue into DNA containing a cisplatin adduct (Ohndorf et al., 1999) is reminiscent of the invasion by an amino acid residue of AAG into DNA containing a modified AP site (Lau et al., 1998). In view of these observations, we studied the interactions of the human AAG protein with cisplatin adducts in a duplex DNA substrate. We used site-specifically modified oligonucleotides, containing each of the cisplatin intrastrand crosslinks, in binding assays with purified human AAG protein, and we demonstrated that there is indeed a tight interaction between the cisplatin adducts and AAG. Furthermore, the repair of εA was measured in the presence of cisplatin adducts, and it was observed that the presence of cisplatin adducts inhibited the excision of εA by AAG. This work provides evidence that DNA repair proteins that become non-productively engaged in complexes with pseudo substrates become significantly less effective in their role to defend cells against important DNA damaging agents.

B. Materials and Methods

Enzymes and Chemicals

The 5'-dimethoxytrityl-etheno-deoxyadenosine 3'-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite was purchased from Glen Research. PAC-dA β-cyanoethyl, i-Pr-PAC-dG β-cyanoethyl, iBU-dC β-cyanoethyl, and T β-cyanoethyl phosphoramidites were obtained from

Amersham Pharmacia Biotech. Cisplatin, *trans*-diamminedichloroplatinum(II) (*trans*-DDP), and

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were from Sigma Chemical Co., methyl

methanesulfonate (MMS) was from Aldrich, and MMC was from Boehringer-Mannheim. Cisplatin

and *trans*-DDP were dissolved in phosphate buffered saline or H_2O, MNNG was dissolved in citrate

buffer pH 5.5, and MMC was dissolved in H_2O. T4 polynucleotide kinase was obtained from New

England Biolabs, and γ-^{32}P ATP was from New England Nuclear Life Sciences. The purified human

AAG protein and the *E. coli* AlkA and Tag proteins were a gift from Dr. Thomas Ellenberger

(Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School,

Boston, MA). The human AAG protein lacks residues 1-79 but its enzymatic activity and DNA

binding specificity are identical to those of the full length protein (Lau et al., 1998).

DNA Substrates

Oligonucleotides were obtained from Research Genetics and purified by polyacrylamide gel

electrophoresis. Platination reactions were carried out in 5 mM Na_3PO_4 buffer pH 7.4 at 37 °C for

18-21 hrs and platinated DNA was purified on denaturing polyacrylamide gels. The platination sites

were confirmed by Maxam-Gilbert sequencing (Lemaire et al., 1991; Brabec and Leng, 1993). The

oligonucleotide containing εA was synthesized on an Applied Biosystems 391 automated DNA

synthesizer using standard phosphoramidite chemistry. The εA oligonucleotide was deprotected in

10% DBU in methanol at room temperature for 48 hrs (Litinski et al., 1997) and purified by

polyacrylamide gel electrophoresis. The sequences of the DNA duplexes are shown in Figure 2.1.

Concentrations were determined by measuring the A_{260} and calculating the extinction coefficients

as described (Borer, 1975).

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Bacterial Strains

The relevant phenotypes of the strains used in this study are shown in Table 2.1. *uvrB* derivatives were made by P1*vir* transduction according to Miller (Miller, 1992) and screening tetracycline-resistant isolates for UV sensitivity. All strains were transformed with a pUC19 plasmid expressing human AAG according to Chung (Chung et al., 1989). Briefly, the cells were spun down at 5,000 g, resuspended in 1xTSS buffer (10% polyethylene glycol, 5% DMSO and 0.05 M MgCl$_2$ in LB), and incubated with plasmid on ice for 30 min. The plasmid was a gift from Dr. Leona Samson (Harvard School of Public Health, Boston, MA).

Strain	Relevant genotype	Source of derivation
GC4801	Wild type (AB1157 background)	S.Boiteux (Boiteux et al., 1992)
GC4803	X::*Tn5 tagA1, alkA1*	S.Boiteux (Boiteux et al., 1992)
GC4801 *uvrB*	*uvrB5*	This work
GC4803 *uvrB*	X::*Tn5 tagA1, alkA1, uvrB5*	This work

Table 2.1: Genotypes of *E. coli* strains used in this study.

Culture of Mammalian Cells

$Aag^{+/+}$ (AB1 and clone 33) and $Aag^{-/-}$ (clone 29 and clone 38) embryonic stem (ES) cells were obtained from Dr. Leona Samson (Harvard School of Public Health, Boston, MA) and have been previously described (Engelward et al., 1996). They were maintained in 15% fetal bovine serum, 2 mM L-Glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 100 μM β-mercaptoethanol, 100 μM non-essential amino acids and murine leukemia inhibitory factor purified according to Mereau (Mereau et al., 1993).

Gel Mobility Shift Assay

Binding assays were carried out in assay buffer containing 4 mM Tris-Cl buffer, pH 7.8, 6 mM Hepes-KOH buffer, pH 7.8, 20 mM KCl, 30 mM NaCl, 0.43 mM EDTA, 1 mM β-mercaptoethanol, 10 ng chicken erythrocyte DNA, 13% glycerol, 360 nM purified human AAG, and 0.2 nM ^{32}P-labeled oligonucleotide. The reaction mixtures were incubated at 20 °C for 15 min and were then electrophoresed on a 6% polyacrylamide gel in 1xTAE buffer at 150 V for 120 min at 4°C.

Competition Experiments

Competition assays were carried out by titrating increasing amounts of unlabeled competitor DNA into binding reactions that contained 61 nM purified human AAG protein and 0.2 nM of the ^{32}P-labeled, εA oligonucleotide. Amounts of bound and free ^{32}P-labeled probe were determined by quantitative analysis of dried gels using a Molecular Dynamics PhosphorImager. The equation used to determine the binding affinity of the protein to the different probes was:

$$\Theta = \frac{1}{2Tt}\left[Kt + \left(\frac{Kt}{Kc}\right)Ct + Tt + Pt - \sqrt{\left[Kt + \left(\frac{Kt}{Kc}\right)Ct + Tt + Pt\right]^2 - 4TtPt} \right] \qquad \text{Equation 1}$$

where Θ is the fraction of bound ^{32}P-labeled εA oligonucleotide, Pt, Tt, and Ct are the concentrations of the protein, ^{32}P-labeled εA oligonucleotide and competitor DNA respectively, and Kt and Kc represent the apparent dissociation constants for the εA, and competitor DNA oligonucleotides,

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respectively (Lin and Riggs, 1972; Long and Crothers, 1995). Competition curves were fit for the best value of Kc by nonlinear least-squares analysis. Relative DNA binding affinities were determined by comparing values of Kc for the competitor oligonucleotides. Experiments with each competitor DNA were performed twice.

Glycosylase Assays

Glycosylase assays were performed at 37 °C in assay buffer containing 20 mM Tris-Cl buffer, pH 7.8, 100 mM KCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 1 mM EGTA, 80 nM to 2.5 μM of unlabeled competitor DNA, 0.7 to 1.1 nM ^{32}P-labeled DNA, and 11-14 nM human AAG. The DNA was enzymatically (50 nM E. coli formamidopyrimidine DNA glycosylase) or chemically (0.1 N NaOH) cleaved at AP sites, and the reaction products were analyzed on a 20% denaturing gel. Amounts of full length substrate and cleaved product were determined by quantitative analysis using a Molecular Dynamics PhosphorImager.

Assessment of Cytotoxicity in Mammalian Cells

Various dilutions of ES cells were seeded onto feeder coated 6-well plates. Sixteen to eighteen hours later, cells were incubated for 1 hr in drug-containing serum-free medium. After 8 days, colonies were dried, fixed in ethanol:acetic acid 3:1, stained with crystal violet, and counted. Survival was determined after correction for the dilution factor. For drug combination experiments, control survival curves were performed for each drug individually. Predicted additive effect was defined as the geometric addition of survivals related to each individual drug.

Assessment of Toxicity in Bacteria

Cells were grown in LB broth to 1-2 x10^8 cells/ml, spun down and resuspended in M9 salts. They were then exposed to various drug concentrations for 1 hr at 37°C. Exposure was stopped by diluting the cells in M9 salts. Appropriate dilutions of the cell suspensions were plated on LB plates and allowed to grow overnight at 37°C. The number of colonies were counted and survival was determined. For the adaptive response induction experiments, cells were exposed to 0.1 μg/ml MNNG for 90 min and to 0.4 μg/ml MNNG for 60 min. We confirmed that the adaptive response was induced by treating adapted wild type cells with MMS or MNNG and showing that their survival was higher than the survival of non-adapted cells (data not shown).

C. Results

Human AAG Recognizes the Various Cisplatin Intrastrand Crosslinks

To test the hypothesis that 3-methyladenine DNA glycosylases might be involved in the repair of cisplatin adducts, we initially studied the interactions of the human AAG protein with the cisplatin intrastrand crosslinks by using a gel mobility shift assay. DNA duplexes containing each of the three intrastrand crosslinks formed by cisplatin were constructed, ^{32}P-labeled, and used in binding reactions with purified human AAG protein. There was substantial binding of the protein to the DNA probes containing the intrastrand crosslinks, as shown by the presence of a slower migrating band (Figure 2.2A lanes 6, 8 and 10) not seen when these duplexes were analyzed in the absence of protein (Figure 2.2A lanes 5, 7 and 9). For comparison, the binding of the human AAG protein to a duplex oligonucleotide containing a single εA adduct was investigated under identical experimental conditions. This probe was readily bound by the human AAG protein (Figure 2.2A

lane 2), as previously described (Dosanjh et al., 1994b; Singer et al., 1992; Roy et al., 1998). In contrast, little binding was observed when unmodified duplex DNA was used in the binding reaction (Figure 2.2A lane 4). Consistent with its action on double stranded DNA, no binding of AAG to εA or to cisplatin DNA adducts was observed when the lesions were located in single stranded DNA (data not shown).

Preliminary experiments showed that mouse Aag was also able to recognize the 1,2-d(ApG) cisplatin adduct, the 1,2-d(GpG) adduct (to a lesser extent) but not the 1,3-d(GpTpG) adduct (data not shown).

To test whether there were a direct involvement of AAG in the repair of cisplatin adducts we tested for glycosylase activity of the protein towards the adducts *in vitro*. No glycosylase activity of the human AAG protein towards any of the oligonucleotides containing the cisplatin intrastrand crosslinks was observed (Figure 2.2B lanes 6, 8, 10, 14, 16 and 18); in contrast, under these experimental conditions εA was efficiently excised by the enzyme (Figure 2.2B lane 2), confirming that the protein was active.

Human AAG Binds Specifically to the Cisplatin Intrastrand Crosslinks

To determine quantitatively the binding affinity of the protein for the various cisplatin DNA adducts, increasing amounts of unlabeled competitor DNA were titrated into binding reactions of the human AAG protein with the ^{32}P-labeled εA oligonucleotide. A representative gel showing the results of such an experiment is shown in Figure 2.3. Addition of unlabeled 1,2-d(ApG) (Figure

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2.3A), 1,2-d(GpG) (Figure 2.3B), or 1,3-d(GpTpG) (Figure 2.3C) oligonucleotides competes away

the binding of the human AAG protein to the εA oligonucleotide. A plot of the average of two

experiments for each competitor DNA is shown in Figure 2.4A. The data were fit to a competitive

binding equation (Equation 1) and the apparent dissociation constants are presented in Figure 2.4B.

The 1,3-d(GpTpG) adduct ($K_{d(app)}$ = 144 nM) was recognized by AAG with comparable

affinity to the 1,2-(GpG) ($K_{d(app)}$ = 115 nM) and 1,2-d(ApG) adducts ($K_{d(app)}$ = 71 nM). This is in

contrast to HMG domain proteins, which recognize the 1,2-intrastrand crosslinks but not the 1,3-

d(GpNpG) crosslink (Pil and Lippard, 1992; Chow et al., 1994).

Interestingly, even though the two 1,2-intrastrand crosslinks of cisplatin bend and unwind

the DNA helix to the same extent (Bellon et al., 1991), the 1,2-d(ApG) crosslink appears to be

recognized more efficiently than the 1,2-d(GpG) crosslink, indicating that the two adducts might

distort the DNA helix differently at the nucleotide level. Indeed, chemical reactivity studies suggest

that the adducted adenine: thymine base pair in the 1,2-d(ApG) crosslink is more distorted than the

corresponding adducted guanine: cytosine base pair in the 1,2-d(GpG) crosslink (Schwartz et al.,

1989). Further support for the notion that the two adducts have distinct structural features comes

from studies with RNA and DNA polymerases. The 1,2-d(GpG) adduct impedes the single step

addition reaction by either the *E. coli* RNA polymerase or the wheat germ polymerase II more

effectively than the 1,2-d(ApG) adduct (Corda et al., 1992), and the 1,2-d(GpG) crosslink is more

efficient than the 1,2-d(ApG) crosslink at inhibiting replication by DNA polymerases T7, Taq and

polymerase I (Comess et al., 1992) indicating that the polymerases can distinguish between the two

structurally similar crosslinks. Finally, the 1,2-d(ApG) crosslink is 4-5 times more mutagenic than the 1,2-d(GpG) crosslink (Yarema et al., 1995).

Cisplatin Adducts Titrate AAG from its Natural Substrates

On the basis of the high affinity of the human AAG protein for the cisplatin intrastrand crosslinks, we hypothesized that the excision of εA by AAG would be inhibited in the presence of the cisplatin adducts. To that end, 0.7 nM of ^{32}P-labeled εA oligonucleotide was incubated with 11 nM human AAG and 2500 nM of unlabeled competitor DNA, and cleavage of the ^{32}P-labeled εA oligonucleotide was measured over time. Unmodified DNA had no effect on the kinetics of cleavage of the ^{32}P-labeled εA oligonucleotide (Figures 5A and 5B lanes 1-6 and data not shown). In contrast, the presence of unlabeled εA (Figure 2.5A lanes 7-12) or 1,2-d(ApG) (Figure 2.5A lanes 13-18) oligonucleotide essentially abolished the ability of AAG to release εA from the ^{32}P-labeled εA oligonucleotide. The 1,2-d(GpG) and 1,3-d(GpTpG) oligonucleotides were also able to inhibit εA excision (Figure 2.5B lanes 7-12 and lanes 13-18, respectively), albeit to a lesser degree, in accordance with the lower affinity of human AAG for these adducts.

The cleavage of ^{32}P-labeled εA oligonucleotide by human AAG was also measured in the presence of varying concentrations of competitor DNA. Unmodified DNA was unable to inhibit the excision of εA by AAG (Figure 2.6A lanes 2-7). In contrast, no cleavage of the ^{32}P-labeled εA oligonucleotide was observed even when low amounts of unlabeled εA competitor DNA were present in the reactions (Figure 2.6A lanes 8-13). When the 1,2-d(ApG) oligonucleotide was used

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as the competitor, there was an inverse correlation between the concentration of competitor DNA and the amount of cleavage of the ^{32}P-labeled εA oligonucleotide (Figure 2.6A lanes 14-19). High concentrations of the 1,2-d(GpG) and the 1,3-d(GpTpG) oligonucleotides were also able to inhibit εA excision by AAG (Figure 2.6B). Interestingly, the 1,3-d(GpTpG) oligonucleotide was not very efficient at inhibiting εA excision corresponding to the lower binding affinity of AAG for this adduct. Increasing the AAG concentration in the repair reactions resulted in higher levels of excision of the εA lesion. Furthermore, this effect was reversed by increasing the concentration of competitor DNA in the repair reactions (data not shown).

While significant inhibition of εA cleavage by AAG in the presence of cisplatin adducts was observed at modest ratios of cisplatin adducts to εA adducts, maximum inhibition was observed when a 2,500 fold molar excess was used in the reactions. Even in this most conservative case, we believe that the presence of cisplatin adducts may be physiologically relevant with regard to the response of a cancer patient to cisplatin based chemotherapy. About 50,000 cisplatin adducts per cell are typically observed in cisplatin treated patients (Reed et al., 1993), whereas approximately 1-20 εA adducts per cell are detected in normal human liver tissue (Misra et al., 1994; Nair et al., 1995; Bartsch et al., 1994), suggesting that repair inhibition might also be operative in a clinically relevant context.

Cisplatin and MMS Act Synergistically to Kill Mammalian Cells

The use of cisplatin in cancer chemotherapy is limited because the doses required to treat certain "resistant" tumors lead to severe multi-organ toxicities (Chu et al., 1993). Since the side

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effects of cisplatin do not overlap greatly with those of other drugs, it is possible to potentiate

cisplatin toxicity without increasing its side effects by administering cisplatin in combination with

other chemotherapeutic agents. Interestingly, cisplatin acts synergistically with several drugs

(Bergerat et al., 1979). In most cases, however, the basis of synergy is not well understood

precluding the rational design of new and effective regimens. We propose that cisplatin adducts

titrate human AAG away from lesions such as 3-mA and εA, leading to enhanced toxicity because

of the inhibition of repair of these lesions by AAG (see discussion). Consequently, we expect

cisplatin to have a synergistic effect in potentiating the toxic effects of agents that introduce in DNA

3-mA, εA or other lesions that are substrates for AAG. To test this hypothesis we treated AB1 ES

cells with cisplatin and MMS in combination, and measured survival. AB1 cells treated with

cisplatin and MMS are more sensitive than predicted based on the toxicity of either drug alone

(Figure 2.8) consistent with our hypothesis.

Mouse ES Cells Deficient in Aag Might Be More Sensitive to Cisplatin

To determine whether mammalian 3-methyladenine DNA glycosylases are involved in

protecting cells from cisplatin cytotoxicity, $Aag^{+/+}$ and $Aag^{-/-}$ mouse ES cells were treated with MMS

or cisplatin and survival was determined. MMS was more toxic to $Aag^{-/-}$ cells than $Aag^{+/+}$ cells

(Figure 2.8A) as previously reported (Engelward et al., 1996; Elder et al., 1998). Interestingly, one

of the $Aag^{-/-}$ clones (clone 29) was more sensitive to cisplatin than the wild type cells whereas the

other $Aag^{-/-}$ clone (clone 38) was as sensitive as the wild type (Figure 2.8B). The reason for this

behavior is not clear but it might be due to differentiation of clone 38. Undifferentiated ES cells

express high levels of wild type p53 and are more sensitive to UV radiation than differentiated cells

that have lower levels of p53, most of which is in the mutant form (Sabapathy et al., 1997). To test

whether differentiation and/or loss of p53 in clone 38 is responsible for the difference in response

between the two null clones, all the clones need to be tested for UV sensitivity and p53 status.

***Bacterial Cells Deficient in 3-mA Glycosylase Activity Do Not Show Enhanced Sensitivity to
Cisplatin***

To determine whether *E. coli* 3-methyladenine glycosylases are involved in the repair of

cisplatin adducts, we measured survival of wild type, *alkA tag*, *uvrB*, and *alkA tag uvrB* cells after

treatment with MMS or cisplatin. Interestingly, *uvrB* cells were more sensitive to MMS than wild

type cells (Figure 2.9A), suggesting that nucleotide excision repair might be involved in the repair

of alkylation damage. Even though this has not been reported previously for the *E. coli* system,

experiments with *S. cerevisiae* cells have demonstrated that *rad1* and *rad4* cells are more sensitive

to MMS than wild type cells (Xiao and Chow, 1998). The *alkA tag* cells were more sensitive to

MMS than wild type cells (Figure 2.9B), reflecting the major role of the 3-methyladenine

glycosylases in the repair of alkylation damage. Furthermore, *alkA tag uvrB* cells were more

sensitive to MMS than *alkA tag* cells (Figure 2.9C), indicating that the role of nucleotide excision

repair in alkylation damage repair is independent of base excision repair.

Wild type and *alkA tag* cells were equally sensitive to cisplatin (Figure 2.10A), as expected

since nucleotide excision repair is operative in these cells. To determine whether the effects of AlkA

and Tag could be unmasked in nucleotide excision repair deficient cells, we compared the toxicity

of cisplatin in *uvrB* and *alkA tag uvrB* cells. The *uvrB* cells were at least 20 fold more sensitive than

the corresponding wild type cells (Figure 2.10B), reflecting the major role of nucleotide excision repair in the repair of cisplatin adducts. The *alkA tag uvrB* cells were slightly more sensitive to cisplatin than the *uvrB* cells (Figure 2.10C), suggesting that the *E. coli* 3-mA DNA glycosylases do not play an important role in survival of cisplatin treated cells. Accordingly, purified AlkA protein did not recognize the cisplatin intrastrand crosslinks (Figure 2.11). Moreover, purified Tag protein did not recognize the cisplatin intrastrand crosslinks (data not shown).

Effects of AAG Expression in E. coli Cells

One very interesting aspect of the base excision repair pathway is that glycosylases can remove their substrates from DNA without interacting with the proteins that are involved downstream of the pathway. Consequently, *alkA tag* cells have been used in functional complementation experiments with proteins from other species. Human, mouse, and yeast 3-mA glycosylases are active when expressed as recombinant proteins in *E. coli,* and they can rescue the sensitivity of the *alkA tag* cells to methylating agents (Engelward et al., 1993; Samson et al., 1991; Chen et al., 1989; Chakravarti et al., 1991; O'Connor and Laval, 1991; Santerre and Britt, 1994; Memisoglu and Samson, 1996). To determine whether *AAG* expression could affect MMS and cisplatin toxicity, wild type, *uvrB, alkA tag,* and *alkA tag uvrB* cells were transformed with plasmid DNA expressing the human AAG protein. Expression of *AAG* sensitized wild type and *uvrB* cells to MMS (Figure 2.12A). This effect has been previously reported for *AAG* expression in Chinese hamster ovary cells (Coquerelle et al., 1995), rat 3-mA glycosylase in Chinese hamster ovary cells (Calleja et al., 1999) and *mag1* and *AAG* expression in *S. cerevisiae* (Glassner et al., 1998). It has been suggested that the effect is due to an imbalance in base excision repair; if AAG is

overexpressed, and one of the following steps in base excision repair is the rate limiting step, then more AP sites, single strand breaks, or non-sealed gaps are generated in DNA that can be repaired leading to increased toxicity.

AAG expression in *alkA tag* and *alkA tag uvrB* cells rescued their sensitivity to MMS (Figure 2.12B) as previously reported (Chakravarti et al., 1991; Samson et al., 1991). Interestingly, *AAG* expression did not affect survival of any of the strains tested after treatment with cisplatin (Figure 2.13). However, most of the models we are proposing for the role of AAG in the cellular responses to cisplatin involve the interaction of AAG with other proteins (see discussion), so it was not a surprise that we failed to observe a phenotype in *E. coli* cells. Alternatively, the levels of *AAG* expression in *E. coli* might not have been sufficiently high to see an effect. This possibility, however, is not unlikely because AAG expression rescued *alkA tag* cells from MMS sensitivity.

Induction of the Adaptive Response Greatly Affects Survival of alkA tag Cells

In non-adapted cells, AlkA accounts for less than 10% of the total 3-mA glycosylase activity, whereas in adapted cells, it accounts for over 50% of the total 3-mA glycosylase activity (Karran et al., 1982). To see whether induction of AlkA expression affects survival after treatment with cisplatin, wild type and *alkA tag* cells were exposed to low levels of MNNG in order to turn on the adaptive response. The MNNG dose used was not toxic in wild type cells, and induction of the adaptive response did not affect survival of these cells after treatment with cisplatin (Figure 2.2.14A). Pretreatment with MNNG greatly affected survival of *alkA tag* cells (0.1-1% survival after treatment with MNNG) indicating the major role of AlkA and Tag proteins in repairing MNNG

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induced DNA damage. Interestingly, *alkA tag* cells and *alkA tag uvrB* cells pretreated with MNNG were extremely sensitive to cisplatin (Figure 2.14). Pretreatment with MNNG also sensitized *alkA tag* cells to *trans*-DDP (Figure 2.15A).

To determine whether *alkA tag* cells became extremely sensitive to all DNA damaging agents after pretreatment with MNNG, we measured the survival of adapted and non-adapted cells after treatment with MMC. Wild type cells were more resistant to MMC than *alkA tag* cells (Figure 2.15B), suggesting that AlkA plays a role in the repair of MMC adducts. This has also been observed with mouse Aag$^{+/+}$ and Aag$^{-/-}$ ES cells; mouse Aag protects cells from MMC toxicity (Engelward et al., 1996). Induction of the adaptive response in *alkA tag* cells greatly enhanced survival (Figure 2.15B), implying that one or more of the proteins that gets induced is/are involved in the repair of MMC adducts. Interestingly, induction of the adaptive response in wild type cells does not affect their survival; therefore, the protein that gets induced most likely plays a minor role in the presence of AlkA.

D. Discussion

Cell survival is correlated with the formation and persistence of cisplatin adducts (Reed et al., 1993; Reed et al., 1987; Reed et al., 1986; Bedford et al., 1988). Cells from tumor tissues that are generally more responsive to cisplatin chemotherapy appear to have lower levels of repair of cisplatin adducts (Bedford et al., 1988; Hill et al., 1994; Koberle et al., 1997). Therefore, the differential capacity for cisplatin adduct repair is postulated to be an important determinant of variability in clinical response to cisplatin therapy (Jones et al., 1994). Since altered expression of

cellular proteins might affect adduct repair and survival of the tumor cells, significant effort has been put into the identification of proteins that recognize cisplatin adducts.

In this report we have demonstrated that the human AAG protein recognizes specifically the intrastrand crosslinks formed by cisplatin. The apparent dissociation constants of AAG for the 1,2-d(ApG), the 1,2-d(GpG) and the 1,3-d(GpTpG) crosslinks were 71 nM, 115 nM, and 144 nM, respectively. Cisplatin adducts are the first substrates of the human AAG protein that are recognized but are not excised by the enzyme.

Model for Basis of Recognition of Cisplatin Adducts by Human AAG

The structural similarities between an HMG box protein bound to a cisplatin DNA adduct and human AAG bound to an AP site analogue provide information regarding the mechanism of recognition of the cisplatin adducts by the human AAG protein. HMG1 is a non-histone chromatin associated protein that has two HMG domains designated A and B, each of which can bind to cisplatin adducts. The structure of the domain A of HMG1 bound to a 16-mer containing a site-specific 1,2-d(GpG) cisplatin adduct was recently solved (Ohndorf et al., 1999). The DNA is strongly kinked at the site of the adduct, and a phenylalanine residue at position 37 (Phe37) intercalates into the minor groove of DNA opposite the adducted d(GpG) site. The aromatic side chain of the Phe37 residue stacks onto the 3' adducted guanine. The stacking interaction of the aromatic residue with the guanine contributes significantly to binding since a mutation of Phe37 to alanine greatly diminishes the binding affinity of domain A for the cisplatin adduct (Ohndorf et al., 1999). Interestingly, similar structural characteristics are observed in the crystal structure of the

human AAG protein complexed to a double stranded DNA containing an AP site analogue (Lau et al., 1998). The DNA is kinked at the AP site, and a tyrosine residue at position 162 (Tyr162) intercalates into the minor groove of the DNA causing the AP pyrrolidine residue to rotate into the enzyme active site. We propose that AAG recognizes cisplatin modified DNA with high affinity because Tyr162 can intercalate between the adducted purines and the stacking interaction can stabilize the complex. Further studies with an AAG protein mutated at position 162 are warranted to determine whether Tyr162 is important for binding of the AAG to the cisplatin adducts.

Repair Shielding Model

Ixr1 is a *S. cerevisiae* HMG box protein that also binds specifically to the 1,2 intrastrand crosslinks of cisplatin (Brown et al., 1993). The dissociation constant for Ixr1 binding an oligonucleotide containing a single 1,2-d(GpG) is 250 nM (McA'Nulty et al., 1996). Furthermore, yeast strains deficient for Ixr1 are two to six times more resistant to cisplatin, and these differences in survival can be abolished when survival is determined in the excision repair mutants *rad2*, *rad4* and *rad14* (McA'Nulty and Lippard, 1996). The model advanced to explain these results is that the binding of Ixr1 to the adducts could block the repair of the adducts by preventing the repair complex from recognizing the cisplatin DNA adduct (McA'Nulty and Lippard, 1996). The adducts persist in DNA thereby potentiating the cytotoxic effects of the drug.

The binding affinity of human AAG for the cisplatin adducts is in the same order of magnitude as that of Ixr1, suggesting that AAG might also be able to shield cisplatin adducts from repair (Figure 2.16A). Interestingly, Northern analysis of polyadenylated RNA from different mouse

tissues revealed that the highest *Aag* mRNA levels are seen in the testis (Engelward et al., 1993). Since testicular tumors are very sensitive to cisplatin based chemotherapy, this finding suggests that AAG might play a role in the organotropic specificity of the drug. It is noteworthy, however, that other proteins that bind cisplatin adducts, such as hMSH2, are also abundant in testis (Wilson et al., 1995; Mello et al., 1996). It is possible that the unique sensitivity of the testicular tumors might stem from the presence of multiple proteins that shield cisplatin adducts from repair.

Repair Recruitment Model

Although there is good evidence that proteins that bind but do not repair cisplatin adducts may shield the adducts from repair, it is also possible that AAG might alleviate the toxic effects of cisplatin by facilitating the repair of cisplatin adducts by other repair enzymes. Such a role has been proposed for the *E. coli* photolyase enzyme. *E. coli* photolyase recognizes the 1,2-d(GpG) adduct with high affinity, and the protein stimulates adduct repair by recruiting the nucleotide excision repair system (Ozer et al., 1995). This mechanism is not expected to be operative in testicular tumor cells since studies with these cells have demonstrated that they have low levels of the XPA protein and the ERCC1-XPF endonuclease complex, which are necessary for nucleotide excision repair (Koberle et al., 1999). There are however some *in vitro* data that support the notion that AAG may somehow enhance the repair of the 1,2-d(ApG) crosslink. One such study measured the relative rates of repair of the two 1,2-intrastrand crosslinks by the mammalian excision nuclease. When excision repair is determined after incubation with crude human cell extracts, the 1,2-d(ApG) crosslink is a better substrate than the 1,2-d(GpG) crosslink (Zamble et al., 1996). In contrast, when excision repair is measured after incubation with purified components of the mammalian excision repair

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machinery, the rates of repair of the two crosslinks are indistinguishable (Zamble et al., 1996). These results suggest that there might be a factor in the cell extracts that alters the rate of repair of one or both of the 1,2-intrastrand crosslinks. Since, the 1,2-d(ApG) adduct was recognized more efficiently by AAG than the 1,2-d(GpG) adduct, it is possible that AAG might facilitate the rate of repair of the 1,2-d(ApG) crosslink by conveying this adduct to another repair system (Figure 2.16B).

Repair Factor Hijacking Model

Another model proposed to explain how HMG box proteins mediate cisplatin toxicity is that cisplatin adducts may act as molecular decoys for transcription factors with HMG box domains, such as the human upstream binding factor (hUBF), resulting in disrupted regulation of genes that are critical for cell survival (Treiber et al., 1994; Zhai et al., 1998). Our present finding that repair of εA is inhibited in the presence of cisplatin adducts suggests a new mechanism by which cisplatin adducts may affect cellular homeostasis. We propose that, analogous to "transcription factor hijacking," cisplatin adducts could titrate repair factors such as AAG away from their substrates, resulting in lower repair levels in the cell. In that way, cisplatin treated cells are essentially AAG deficient and therefore less able to tolerate the rigors of multi-drug chemotherapeutic regimens. Consistent with this hypothesis, cisplatin acts synergistically with several other drugs including BCNU (Bergerat et al., 1989).

εA arises in DNA as a result of lipid peroxidation or after exposure to ethyl carbamate or vinyl chloride (Kusmierek and Singer, 1982; Leithauser et al., 1990). The latter compound is used in plastic production, and it has also been detected in BCNU treated patients (Clemens et al., 1982).

It is a human carcinogen that reacts, after metabolic activation, with nucleophilic sites in DNA to form etheno adducts, underscoring the importance of εA repair in preventing mutagenesis and carcinogenesis. We propose that in a cisplatin treated cell the human AAG protein would be in a complex with cisplatin adducts and, therefore, unavailable to repair εA lesions (Figure 2.16C). Furthermore, we expect that the repair of other substrates of AAG, such as 3-mA, would also be inhibited in the presence of cisplatin adducts. 3-mA can block DNA replication, and it can induce sister chromatid exchanges, chromosomal aberrations, cell cycle arrest, and apoptosis (Engelward et al., 1997). Consequently, 3-mA is a toxic adduct that can have detrimental effects if left unrepaired.

E. Conclusions

All of the results detailed above suggest a novel mechanism by which cisplatin can affect cellular homeostasis. We propose that cisplatin adducts "titrate" human AAG away from lesions such as 3-mA and εA, leading to enhanced toxicity because of the inhibition of repair of these lesions by AAG. Consequently, we expect cisplatin to have a synergistic effect in potentiating the toxic effects of agents that introduce in DNA 3-mA, εA or other lesions that are substrates for AAG. These data suggest that cisplatin might be more effective in treating tumors if administered along with a DNA methylating agent or an agent that forms other types of toxic DNA adducts that are substrates for AAG.

F. Future Work

Possible Recognition of a Cisplatin Interstrand Crosslink by AAG

Even though interstrand crosslinks (ICLs) represent a small fraction of the lesions that are formed after reaction of cisplatin with DNA, several studies have demonstrated increased repair of ICLs in cisplatin resistant cell lines, suggesting that the ICLs might be contributing to the cytotoxic effects of cisplatin (Plooy et al., 1985; Meyn et al., 1982; Johnson et al., 1994). Therefore, it is important to determine the pathways that are involved in the recognition and repair of these lesions. ICLs induce distinct structural alterations in the DNA helix, and these alterations might play a role in the recognition of ICLs by cellular proteins. Gel electrophoresis experiments have demonstrated that ICLs unwind the DNA helix by 80° and bend it toward the minor groove by 20-45° (Malinge et al., 1994). Moreover, NMR studies have revealed that the *cis*-diammineplatinum(II) bridge resides in the minor, rather than the major, groove of the duplex and that the complementary deoxycytidines are extrahelical (Huang et al., 1995; Paquet et al., 1996). Interestingly, modeling studies of the structure of several glycosylases complexed with DNA suggest that the glycosylases scan the surface of DNA until they encounter a locally destabilized site containing a lesion (Verdine and Bruner, 1997). The damaged base has to be flipped out of the DNA helix and into the active site of the protein for catalytic chemistry to ensue. This model suggests that structures containing flipped out bases might be recognized by glycosylases. One such substrate could be the structure formed by the interstrand crosslink of cisplatin. Interestingly, the structure of a self-complementary oligonucleotide with two central G:T mismatches in complex with the MUG glycosylase is very similar to the structure of the ICL of cisplatin (Barrett et al., 1998). Therefore, to test the hypothesis that a cisplatin interstrand crosslink might be recognized by AAG, an oligonucleotide containing a

single interstrand crosslink would be constructed and used in binding assays. The synthesis of the interstrand crosslink will be performed in a manner analogous to that previously described in the literature (Brabec et al., 1993).

Mode of Recognition of Cisplatin Adducts by AAG

DNase I footprinting studies have demonstrated that human AAG protects 8 nucleotides on εA containing strand and 16-17 nucleotides in the complementary strand (Roy et al., 1998). Furthermore, the crystal structure of human AAG complexed with DNA containing a transition state mimic demonstrates that AAG has contacts with both DNA strands (Lau et al., 1998). In the absence of a crystal structure of AAG complexed with cisplatin adducts, footprinting studies could be carried out to determine the mode of recognition of cisplatin adducts by AAG and compare it to that for εA lesions. Furthermore, the AAG protein containing a Y162A mutation could be used in binding assays with cisplatin adducts to determine whether intercalation of Tyr162 is important in the recognition of cisplatin adducts. Finally, cisplatin analogues could be used to determine whether AAG recognizes cisplatin adducts through major or through minor groove. Cisplatin and *cis-*dichloroethylenediamineplatinum(II) (en) form the same spectrum of adducts (Eastman et al 1983). The en compound has an extra ethylene bridge in the major groove. If recognition of cisplatin adducts by AAG takes place through the major groove, then substitution of cisplatin adducts with en adducts would result in a reduction in the amount of binding.

Host Cell Reactivation of Plasmid DNA Damaged with Cisplatin

The host cell reactivation assay has been very useful in the study of the repair of damaged

DNA in a mammalian cell (Sheibani et al., 1989; Henderson et al., 1989). The assay involves reactivation of a damaged plasmid that contains a reporter gene in a configuration that permits expression in mammalian cells. After transfection of cells with the damaged plasmid, lesions introduced in the gene will abolish expression, and repair of these lesions by the recipient cells will restore expression, thereby providing an efficient system to study DNA repair. To test whether Aag is involved in the repair of cisplatin adducts, a non-replicating plasmid DNA containing a reporter gene like β-gal, CAT, luciferase, or green fluorescent protein gene, damaged with cisplatin could be transfected in $Aag^{+/+}$ and $Aag^{-/-}$ cells and the expression of the gene could be measured over time. If Aag is somehow involved in the repair of cisplatin adducts, then expression of the reporter gene should be restored faster or slower (depending on whether Aag facilitates or inhibits adduct repair) in the $Aag^{+/+}$ cells than in the $Aag^{-/-}$ cells.

The host cell reactivation assay could also be exploited to determine whether inhibition of εA or 3-mA repair by cisplatin adducts can take place *in vivo*. Wild type cells could be transformed with plasmid DNA containing a reporter gene that has been damaged with an alkylating agent and with plasmid DNA containing various levels of cisplatin adducts, and expression of the reporter gene versus cisplatin damage could be measured. If cisplatin adducts can "hijack" AAG away from its natural substrates *in vivo*, we should see a decrease in the level of expression of the reporter gene as we increase the number of cisplatin adducts. This system would probably not be able to determine whether cisplatin adducts "hijack"AAG away from εA since εA is not a blocking lesion.

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Possible Role of Mammalian 3-mA Glycosylases in Cisplatin Cytotoxicity

Since the data on the role of *Aag* in cisplatin cytotoxicity were inconclusive, further studies need to be carried out to determine whether mammalian 3-mA glycosylases mediate cisplatin toxicity. The following include some of the possible experiments designed to address this question.

1. Measure the levels of sister chromatid exchanges (SCEs) and chromosomal aberrations in $Aag^{+/+}$ and $Aag^{-/-}$ cells after treatment with cisplatin. Even though survival of cisplatin treated is sensitive to p53 status of the cells, formation of SCEs and chromosomal aberrations is not thought to be dependent on p53 status.

2. Measure the mutation frequency induced by cisplatin adducts in $Aag^{+/+}$ and $Aag^{-/-}$ cells.

3. Introduce *Aag* gene in $Aag^{-/-}$ cells and see whether cells become more sensitive or more resistant to cisplatin.

4. Measure survival of transformed fibroblasts and/or bone marrow cells from $Aag^{+/+}$ and $Aag^{-/-}$ mice to determine survival of cells after treatment with cisplatin. Furthermore, the survival of $XPA^{-/-}Aag^{+/+}$ and $XPA^{-/-}Aag^{-/-}$ cells could be determined.

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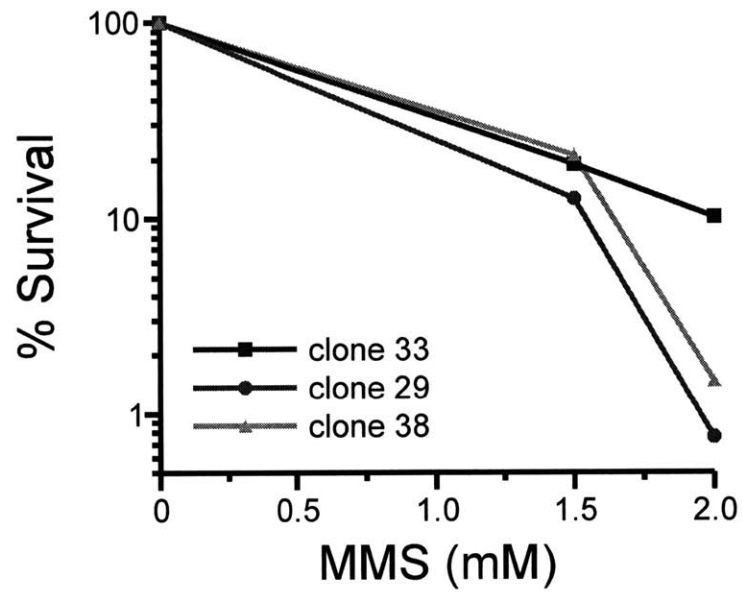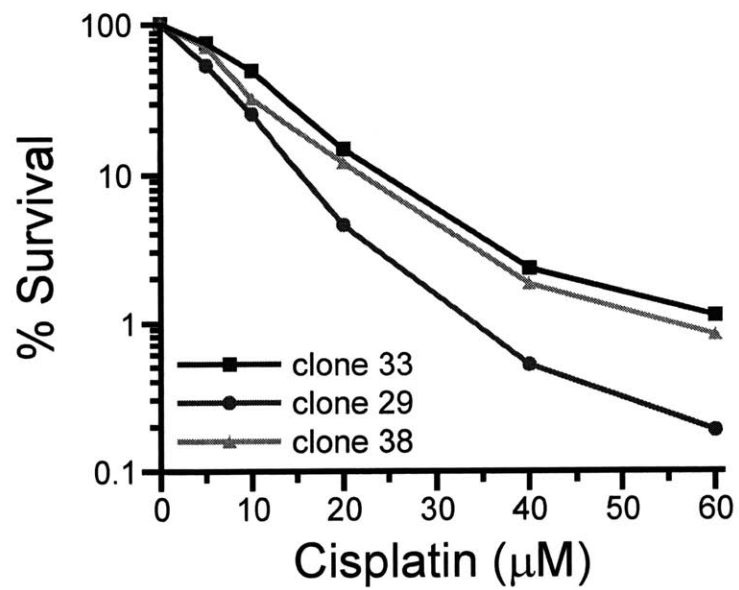
Figure 2.7: Survival curves of *Aag*^{+/+} and *Aag*^{-/-} cells after treatment with increasing doses of **(A)** MMS or **(B)** cisplatin. The data in these graphs represent an average of 4 experiments.
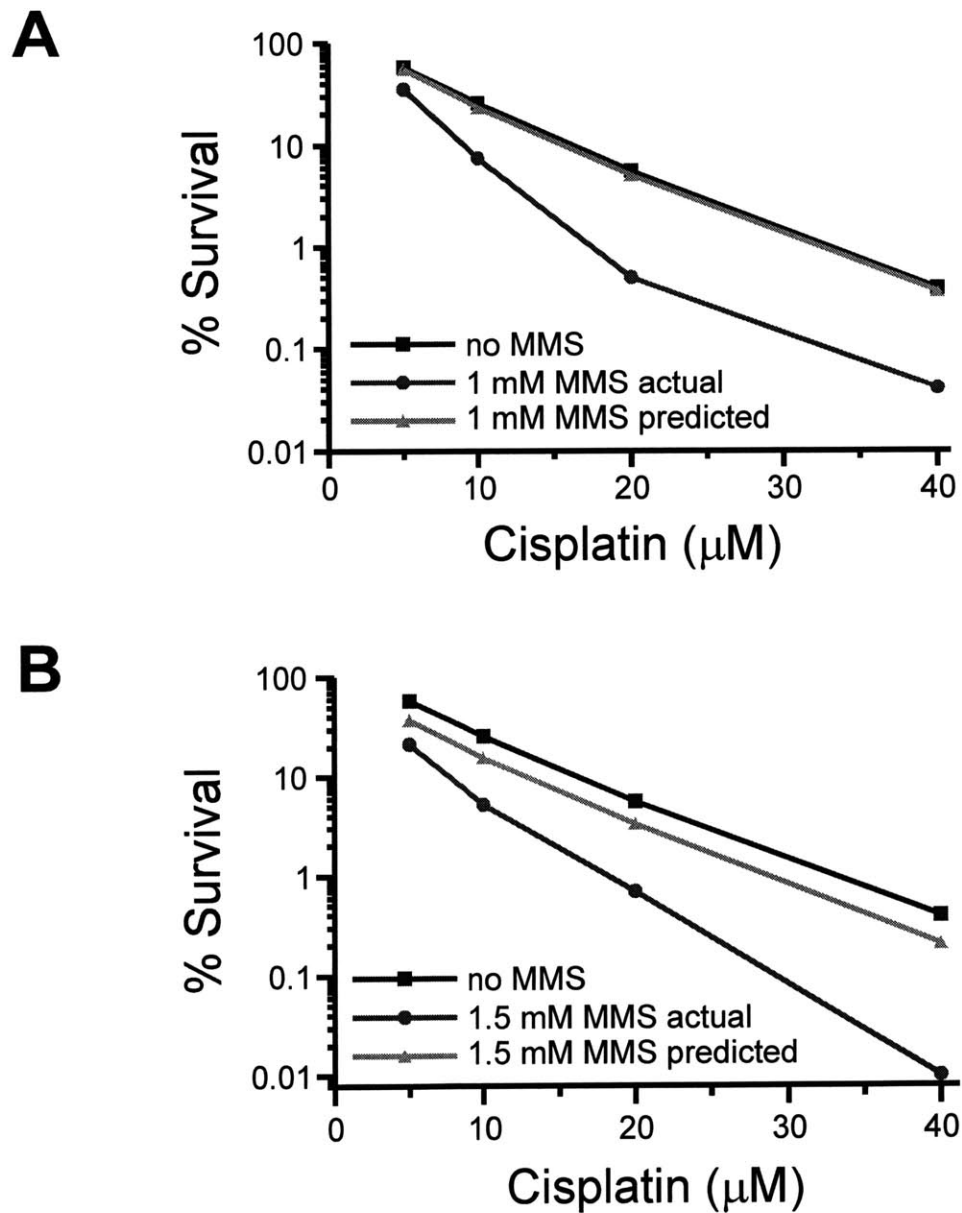
Figure 2.8: Survival curves of AB1 ES cells after treatment with a combination of cisplatin and MMS. **(A)** Survival of cells after treatment with cisplatin alone or w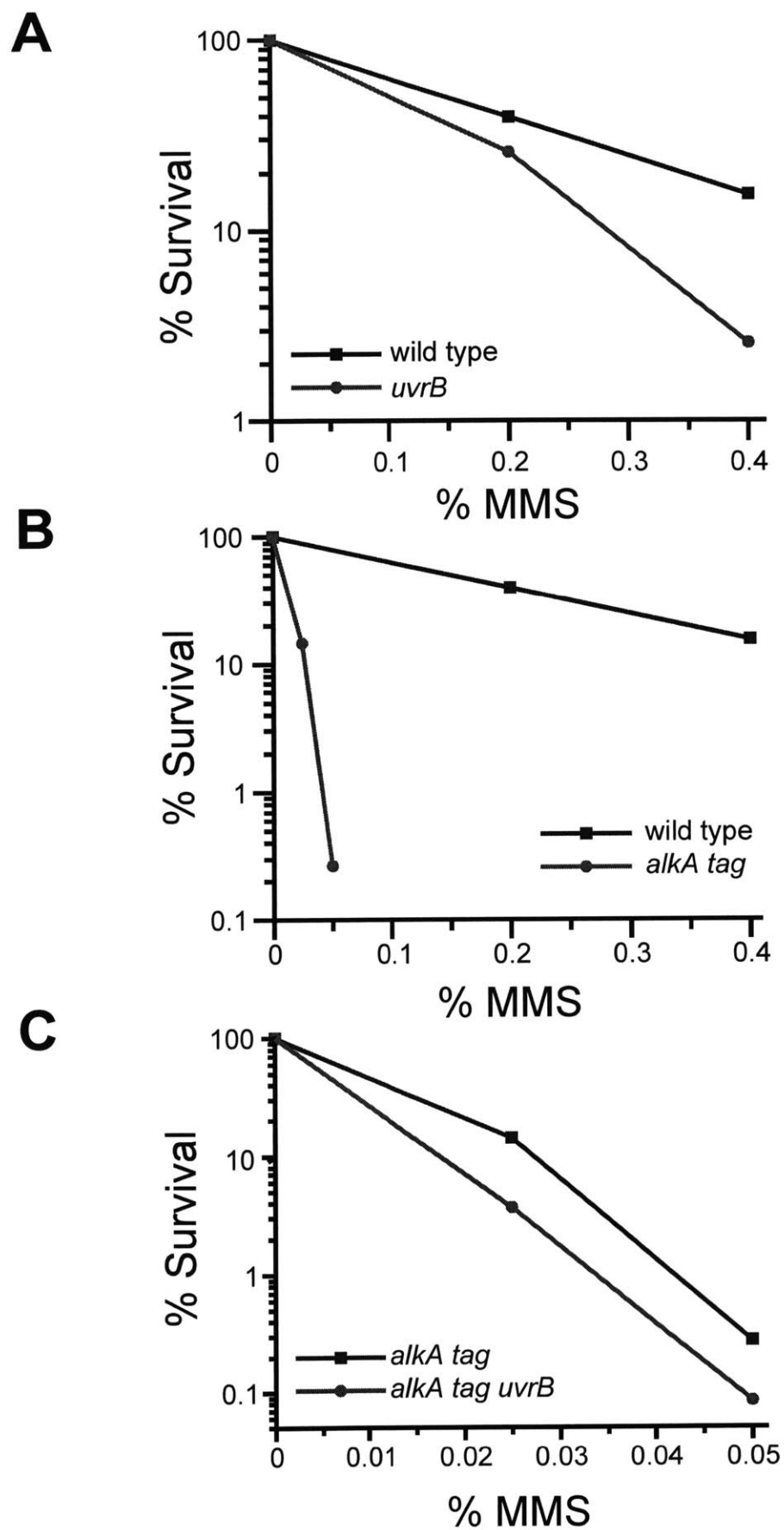ith 1.0 mM MMS. This dose of MMS results in 91.8 % survival. **(B)** Survival of cells after treatment with cisplatin alone or with 1.5 mM MMS. This dose of MMS results in 59.8 % survival.

Figure 2.9: MMS toxicity in **(A)** wild type and *uvrB* cells, **(B)** wild type and *alkA tag* cells and **(C)** *alkA tag* and *alkA tag uvrB* cells. The data in these graphs represent an average of 3 experiments.
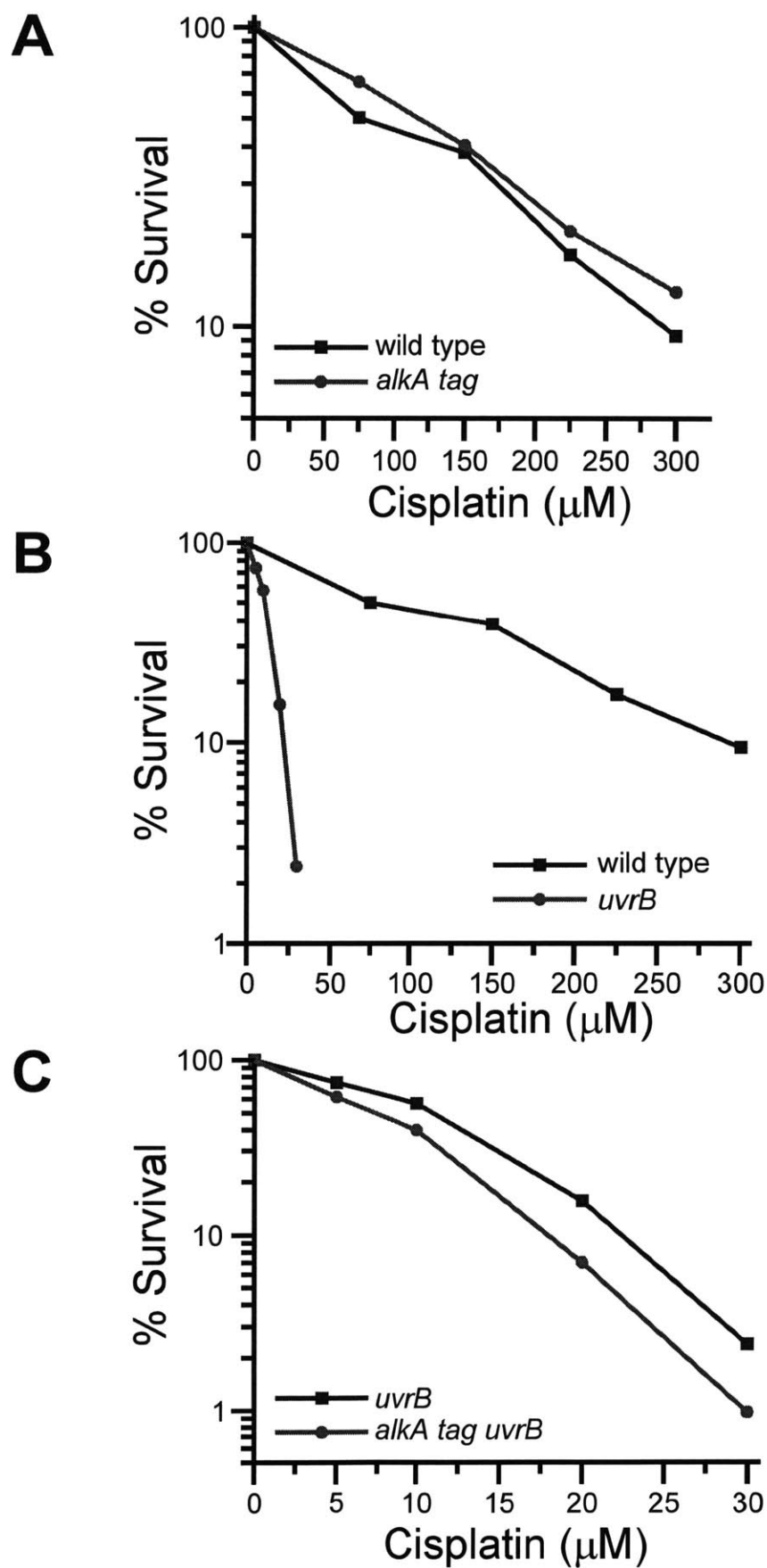
Figure 2.10: Cisplatin toxicity in **(A)** wild type and *alkA tag* cells, **(B)** wild type and *uvrB* cells and **(C)** *uvrB* and *alkA tag uvrB* cells. The data in these graphs represent an average of 3 experiments.
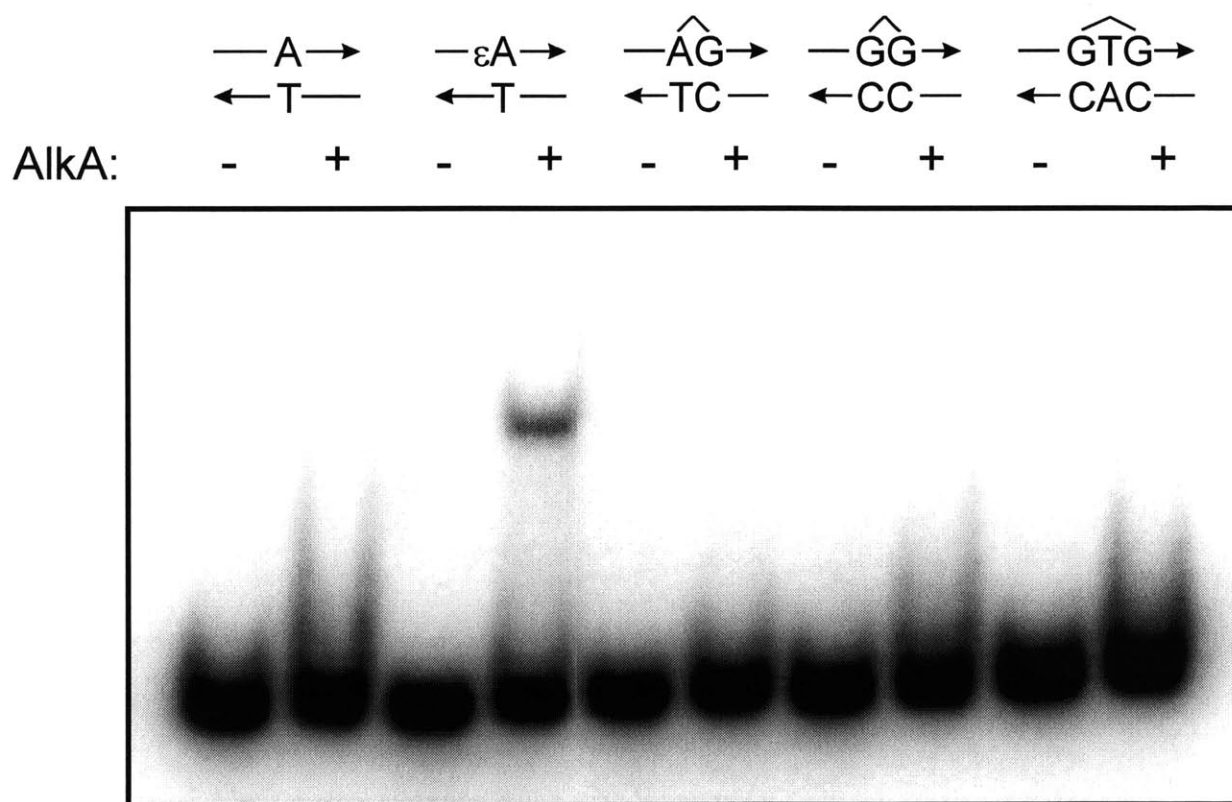
**Figure 2.11:** Gel mobility shift assay of *E. coli* AlkA with various oligonucleotide duplexes. The ^{32}P-labeled oligonucleotides containing a single εA (lanes 3 and 4), 1,2-d(ApG) (lanes 5 and 6), 1,2-d(GpG) (lanes 7 and 8), or 1,3-d(GpTpG) (lanes 9 and 10) were incubated in the presence (+) or absence (-) of 360 ng AlkA. Unmodified DNA (lanes 1 and 2) was used as a control.
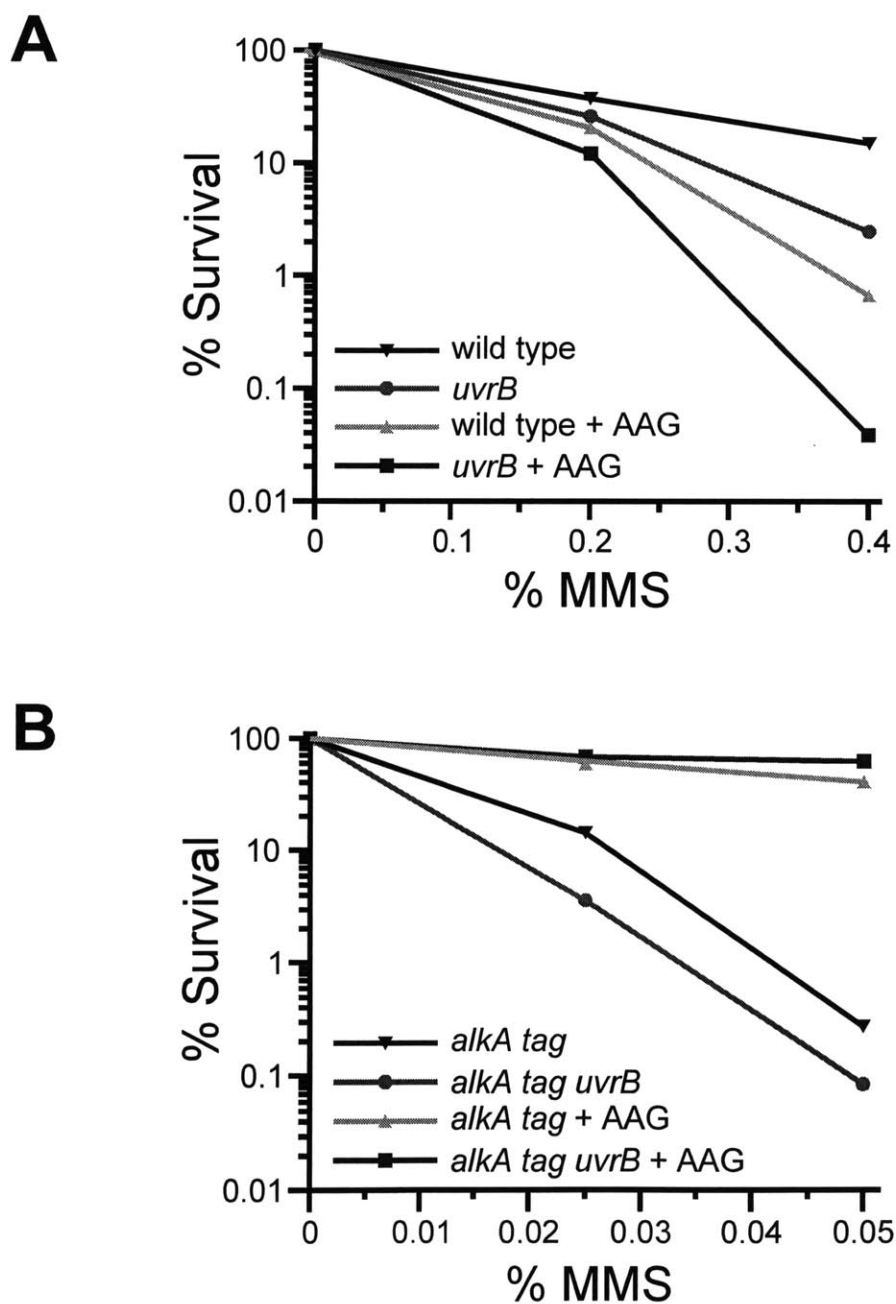
Figure 2.12: Effect of AAG expression on MMS sensitivity of **(A)** wild type and *uvrB* cells and **(B)** *alkA tag* and *alkA tag uvrB* cells. The data in these graphs represent an average of 3 experiments.
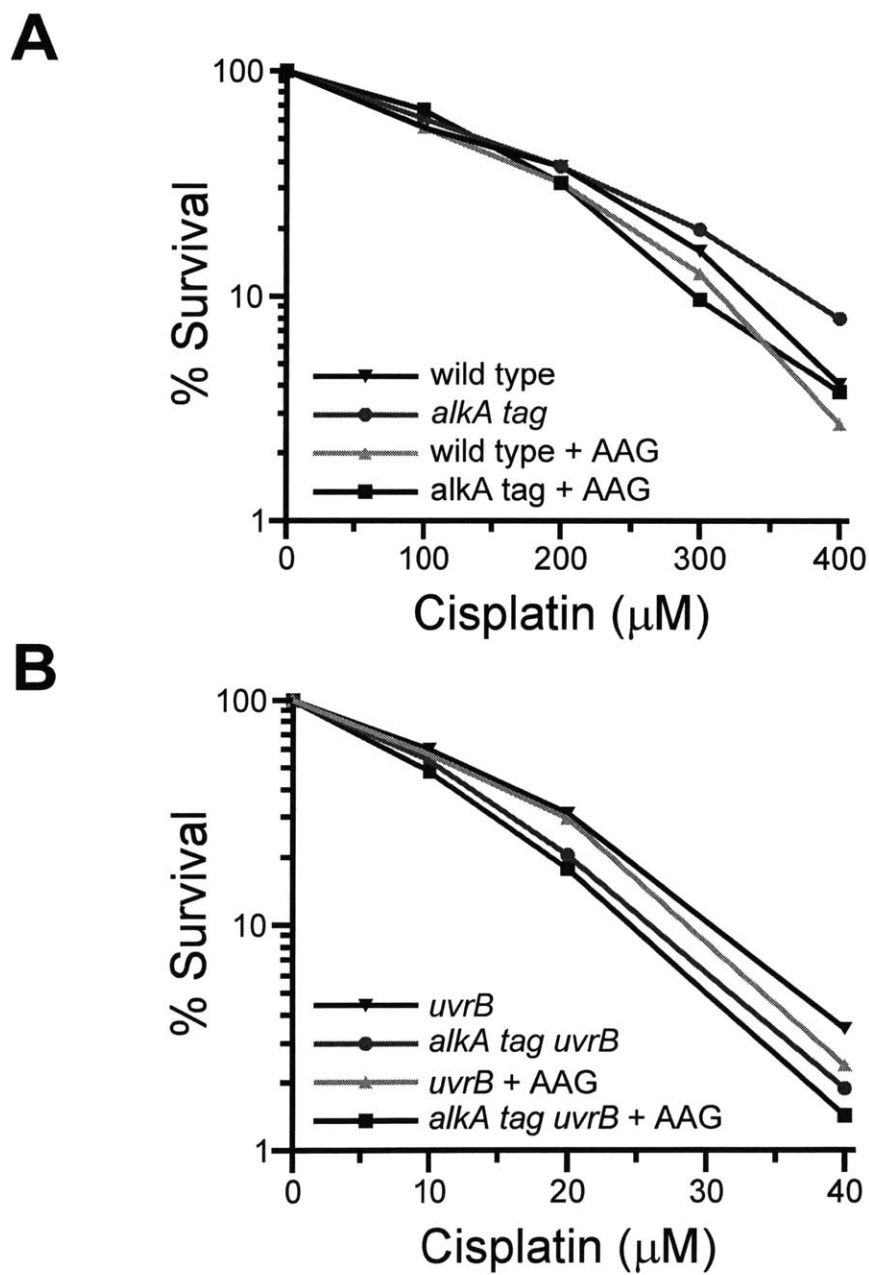
Figure 2.13: Effect of AAG expression on cisplatin sensitivity of (A) wild type and *alkA tag* cells and **(B)** *uvrB* and *alkA tag uvrB* cells. The data in these graphs represent an average of 3 experiments.
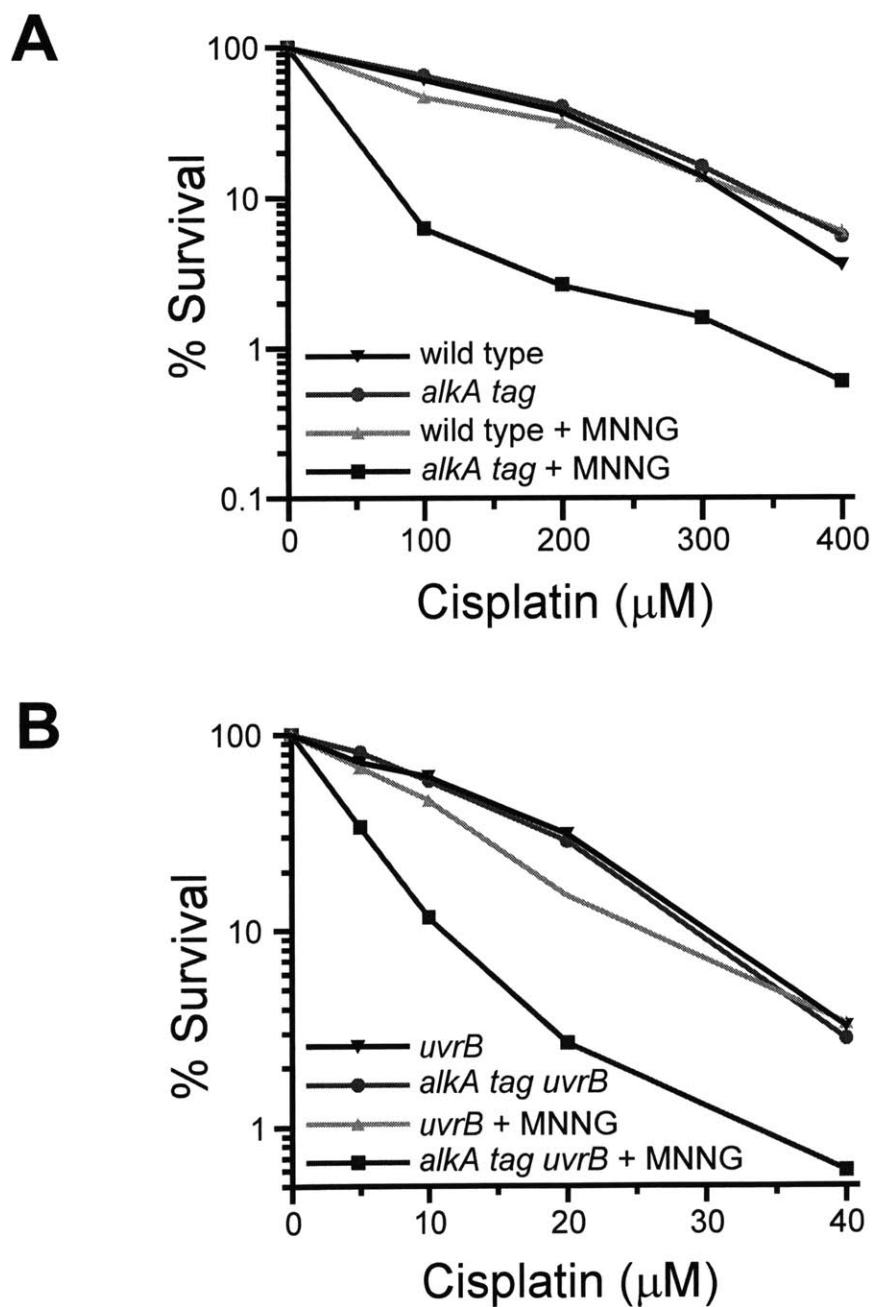
Figure 2.14: Effect of MNNG pretreatment on cisplatin toxicity of **(A)** wild type and *alkA tag* cells and **(B)** *uvrB* and *alkA tag uvrB* cells. The data in these graphs represent an average of at least 4 experiments.
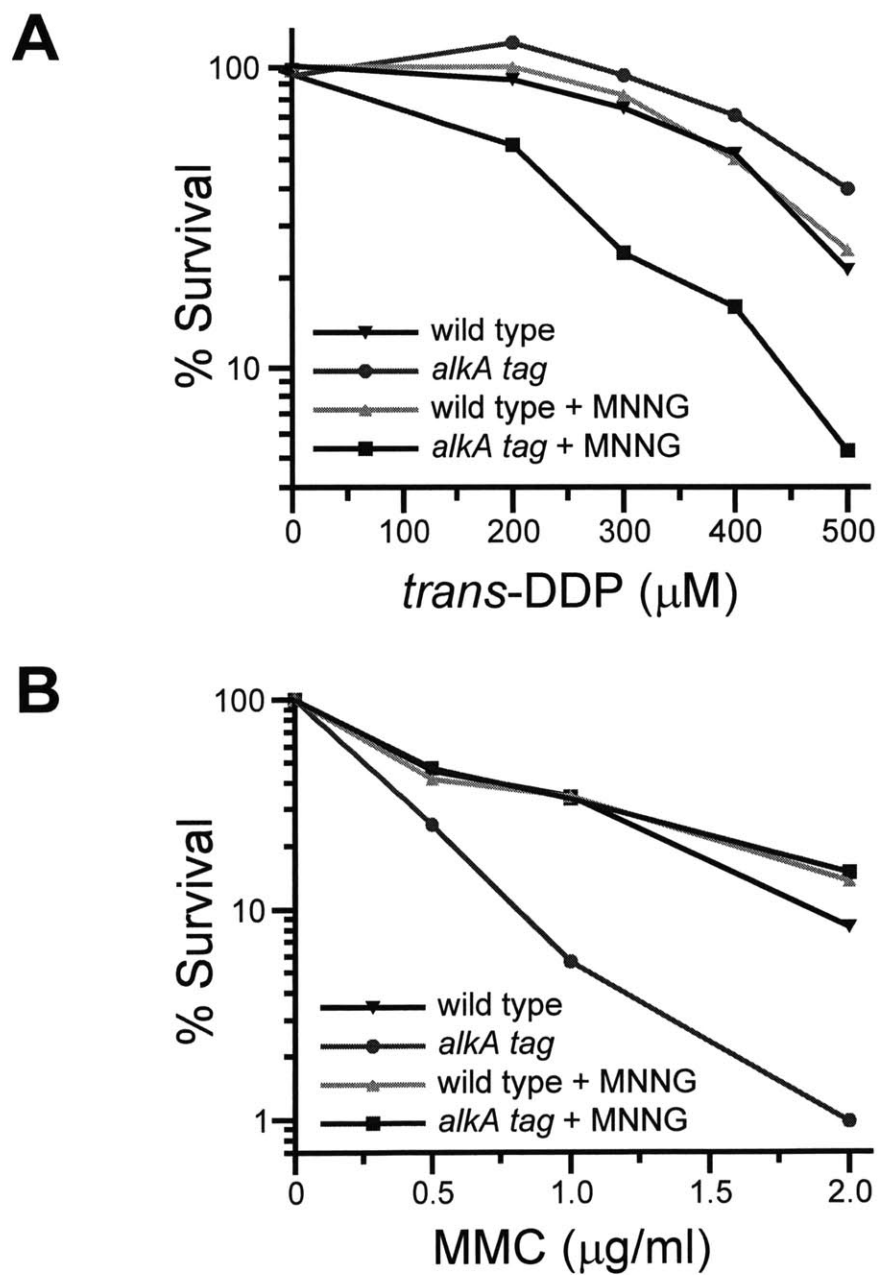
Figure 2.15: Effect of MNNG pretreatment on **(A)** *trans*-DDP and **(B)** MMC toxicity of wild type and *alkA tag* cells. The data in these graphs represent an average of 3 experiments.
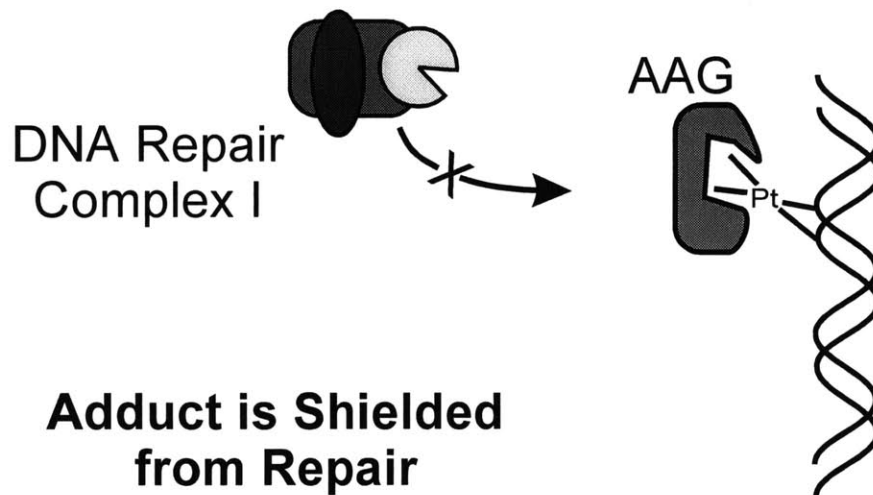
A

Figure 2.16: **(A)** The repair shielding model. Human AAG recognizes cisplatin adducts and can inhibit their repair by physically blocking the access to other repair proteins. The cisplatin adducts persist in DNA thereby potentiating their toxicity.
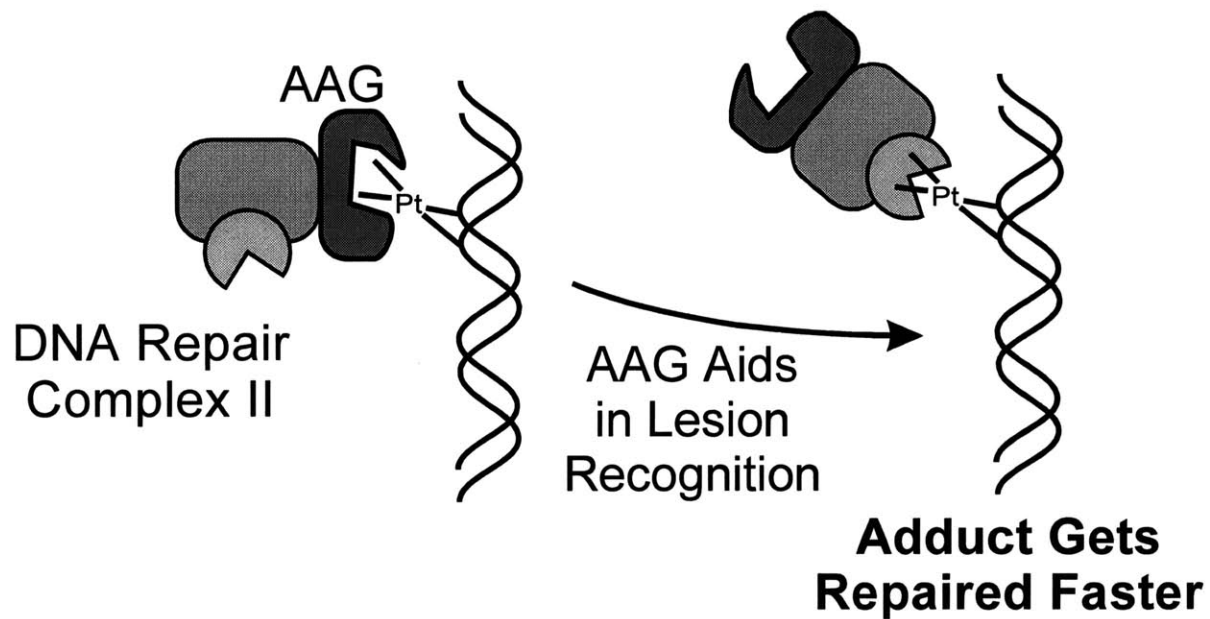
B

Figure 2.16: (B) The stimulation of repair model. AAG recognizes cisplatin adducts and can also interact directly with other repair proteins. AAG can then convey the adducts to the other repair proteins, the adducts are repaired faster and their cytotoxic effects are diminished. The 1,2-d(ApG) is recognized more efficiently by AAG, therefore this mechanism is expected to be more relevant for this adduct.
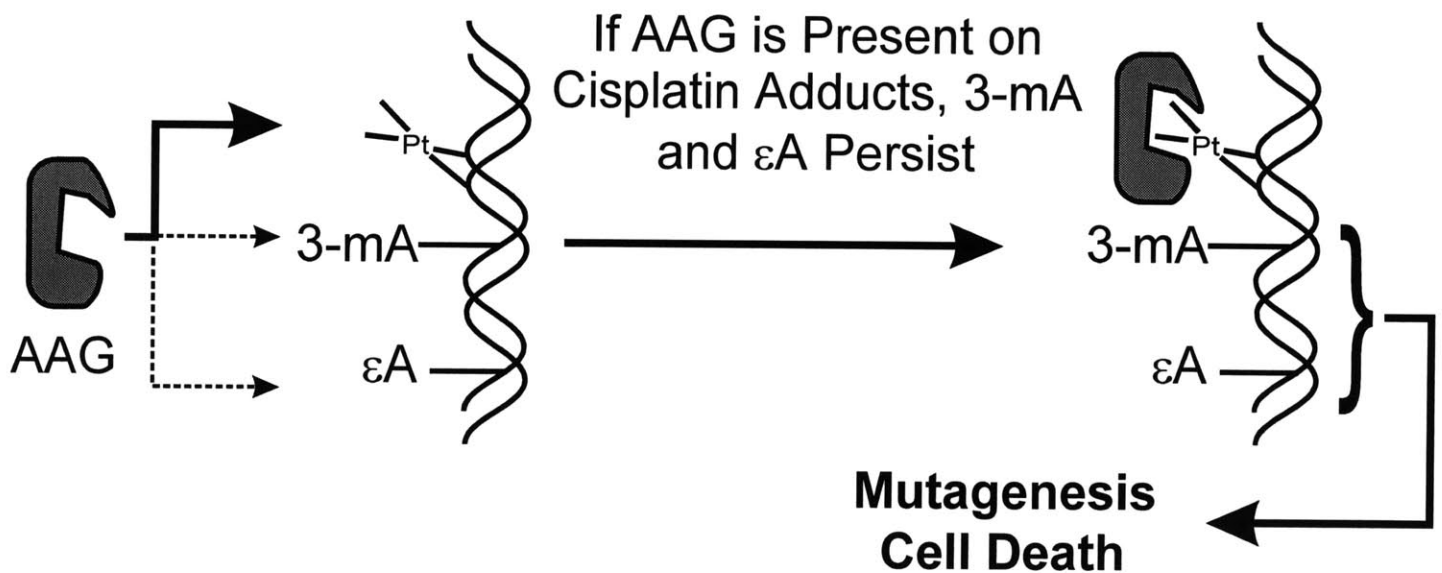
C

Figure 2.16: **(C)** The repair factor hijacking model. Human AAG can interact and repair its normal substrates or it can interact with cisplatin adducts. In a cell treated with cisplatin, the concentration of cisplatin adducts is high enough so that most of the AAG protein would be in a complex with the cisplatin adducts. The repair of the normal substrates of AAG, such as 3-mA and εA, would be inhibited, the lesions would persist in DNA and they could result in mutagenesis, cell cycle arrest and/or programmed cell death.

Chapter 3

The Role of MutM and MutY Glycosylases in the Repair of Cisplatin Adducts

A. Introduction

cis-Diamminedichloroplatinum(II) (cisplatin) is a successful cancer chemotherapeutic agent that is particularly effective in the treatment of testicular tumors, where cisplatin based combination chemotherapy can afford cure rates of greater than 90% (Feuer et al., 1993). The therapeutic effect of cisplatin is thought to be based on the covalent modification of DNA (Bruhn et al., 1990). DNA isolated from cells of patients that have been treated with cisplatin contains approximately 65% 1,2-d(GpG), 25% 1,2-d(ApG), 5-10% 1,3-d(GpNpG) intrastrand crosslinks and a small percentage of interstrand crosslinks and monofunctional adducts (Eastman, 1983; Eastman, 1985; Fichtinger-Schepman et al., 1985; Fichtinger-Schepman et al., 1987). The geometric isomer of cisplatin, *trans*-diamminedichloroplatinum(II) (*trans*-DDP) can also form DNA adducts. *In vitro* studies have demonstrated that *trans*-DDP forms intrastrand crosslinks between two guanine residues or a guanine and a cytosine separated by at least one base, and interstrand crosslinks between complementary guanine and cytosine residues (Brabec and Leng, 1993; Eastman et al., 1988; Eastman and Barry, 1987). Interestingly, *trans*-DDP fails to show any antitumor activity; therefore, the antitumor activity of platinum drugs does not arise from just the interaction with DNA. Instead, the difference in efficacy of platinum drugs has been attributed to the different structural alterations induced in DNA after interaction with the platinum drugs, and the differential recognition of these altered DNA structures by cellular proteins. Of relevance is a family of proteins possessing a high mobility group (HMG box) domain. HMG box proteins display a selective activity for the DNA adducts of therapeutically active platinum compounds (Hughes et al., 1992; Pil and Lippard, 1992; Toney et al., 1989) suggesting a role for such proteins in mediating cisplatin cytotoxicity.

Base excision repair is usually involved in the repair of bases that arise in DNA as a result of deamination, oxidation, or alkylation processes that frequently occur in cells {Friedberg, Walker, et al. 1995 ID: 2173}. DNA glycosylases are an extensive family of enzymes that catalyze the first step of base excision repair. Specifically, they recognize certain DNA bases in DNA and catalyze the hydrolysis of the N-glycosydic bond between the recognized base and the sugar-phosphate backbone (Krokan et al., 1997). The *mutM* and *mutY* genes encode DNA glycosylases involved in base excision repair; collectively, these genes constitute part of the "GO" repair system (Michaels and Miller, 1992) that, heretofore, has been associated with cellular defenses against the oxidative lesion, 7,8-dihydro-oxoguanine (8-oxoG) (Grollman and Moriya, 1993; Michaels et al., 1992). The MutM protein is a bifunctional DNA glycosylase that removes ring-opened purines and 8-oxoG from DNA and can cleave AP sites in DNA (Michaels et al., 1991). The MutY protein removes adenine from A: G, A: C, and A: 8-oxoG mismatches (Au et al., 1989; Michaels et al., 1992; Michaels et al., 1992; Tsai-Wu et al., 1992).

In the first part of this thesis, we have demonstrated that cisplatin adducts can be recognized by a component of base excision repair, namely by the human 3-methyladenine DNA glycosylase. In the present study, we investigated the role of the *mutM* and *mutY* genes in cisplatin toxicity and mutagenicity. Furthermore, the interaction of purified MutM and MutY proteins with cisplatin adducts was determined in gel mobility shift assays. Finally, the role of MutY in the repair of cisplatin adducts was investigated.

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B. Materials and Methods

Enzymes and Chemicals

Cisplatin, *trans*-diamminedichloroplatinum(II) (*trans*-DDP), ampicillin, and rifampicin were from Sigma Chemical Co.. The purified MutM protein was obtained from Dr. J.H. Miller (University of California, Los Angeles) or purchased from Trevigen. The purified MutY protein was either purchased from Trevigen or obtained from Dr. R.S. Lloyd (University of Texas Medical Branch). The purified p26 and D138N p26 fragments of MutY were obtained from Dr. R.S. Lloyd (University of Texas Medical Branch). T4 polynucleotide kinase, uracil glycosylase, Ban I restriction endonuclease, and DNA polymerase I large fragment (Klenow) were obtained from New England Biolabs, and γ-^{32}P ATP and α-^{32}P GTP were from New England Nuclear Life Sciences.

Bacterial Strains and Plasmids

Isogenic *E. coli* strains were obtained from Dr. J.H. Miller (University of California, Los Angeles). Their relevant phenotypes are shown in Table 3.1. *uvrB* derivatives were made by P1*vir* transduction according to Miller (Miller, 1992) and screening tetracyclin-resistant isolates for UV sensitivity. The pKKYEco, pKKYEcop26, and pKKYEcop26D138N plasmids expressing MutY, p26, and D138N p26 respectively, under the control of the *tac* promoter, were obtained from Dr. R.S. Lloyd (University of Texas Medical Branch). *E. coli* cells were transformed with each of the plasmids by electroporation. When indicated in the text, expression of the genes was induced by addition of 100 μM IPTG in the growth medium.

Strain	Relevant Genotype	Source of Derivation
CC104	wild type	(Michaels et al., 1992)
CC104 mutM	mutM::mini-*Tn10*-Kan	(Michaels et al., 1992)
CC104 mutY	mutY::mini-*Tn10*	(Michaels et al., 1992)
CC104 mutM mutY	mutM::mini-*Tn10*-Kan mutY::mini-*Tn10*	(Michaels et al., 1992)
CC104 uvrB	uvrB5::*Tn10*	This work
CC104 mutM uvrB	mutM::mini-*Tn10*-Kan uvrB5::*Tn10*	This work
CC104 mutY + pKKYEco	mutY$^+$	This work
CC104 mutY + pKKYEcop26	p26$^+$	This work
CC104 mutY + pKKYEcop26D138N	D138N p26$^+$	This work

Table 3.1: Genotypes of *E. coli* strains used in this study.

Assessment of Cisplatin Toxicity

Cells were grown in LB broth to 1-2 x10^8 cells/ml, spun down, and resuspended in M9 salts. They were then exposed to various concentrations of cisplatin or *trans*-DDP for 1 hr at 37°C. Exposure was stopped by diluting the cells in M9 salts. Appropriate dilutions of the cell suspensions were plated on LB plates and allowed to grow overnight at 37°C. The number of colonies were counted and survival was determined. Each survival curve was repeated a minimum of 4 times.

Assessment of Mutagenic Potency of Cisplatin

Cells were grown in LB broth to 1-2 x10^8 cells/ml, spun down, and resuspended in M9 salts.

After treatment with various concentrations of cisplatin, appropriate dilutions of the cell suspensions were plated on LB plates and allowed to grow overnight at 37°C to determine survival. Additionally, 50 µl of the treated cells were grown in LB broth overnight. The following day, appropriate dilutions were plated on LB plates to determine the number of viable cells in the cultures, or they were plated on LB plates containing 100 µg/ml rifampicin to determine the number of rifampicin resistant (Rif^R) cells. The ratio of Rif^R cells to the total number of cells after correction for the dilution factor was the mutation frequency. The fold induction was determined as the ratio of the mutation frequency in the treated samples over the mutation frequency in the corresponding untreated control.

DNA Substrates

Oligonucleotides were obtained from Research Genetics, or they were synthesized by standard phosphoramidite chemistry on an Applied Biosystems 391 automatic DNA synthesizer and purified on 15% denaturing polyacrylamide gels. Platination reactions were carried out in 5 mM NaPO$_4$, pH 7.4, at 37°C for 18-21 hours. Platinated DNA was purified on 20% denaturing polyacrylamide gels. Concentrations were determined by measuring the A$_{260}$ and calculating the extinction coefficient as described (Borer, 1975). The sequences of the oligonucleotide duplexes are shown in Figure 3.1. The AP site containing oligonucleotide was prepared by incubating a ^{32}P-labeled duplex oligonucleotide containing a uracil opposite an adenine with 2 units uracil glycosylase for 1 hr at 37°C followed by 18 hrs at 4°C. The complete conversion of uracil to AP site was monitored by treatment of the oligonucleotide with 0.1 N NaOH and analysis of the products in a denaturing polyacrylamide gel.

Restriction enzyme digestion of plasmid pGEM with Ban I yielded a 172 bp fragment that was modified with cisplatin or *trans*-DDP to a bound drug/nucleotide ratio of 0.02. Platination reactions were carried out in 5 mM NaPO$_4$, pH 7.4, at 37°C for 19 hrs. The plasmid DNA was ethanol precipitated and washed three times with 70% ethanol to remove unbound drug.

Gel Mobility Shift Assay

Binding reactions with MutM protein were carried out in assay buffer containing 10 mM Hepes-KOH, pH 7.4, 100 mM KCl, 1 mM EDTA, 10% glycerol, 32 nM MutM protein, and 1 nM DNA. Binding assays with MutY protein were carried out in an assay buffer containing 4 mM Tris-Cl, pH 7.6, 20 mM KCl, 0.4 mM EDTA, 1 mM β-mercaptoethanol, 5 ng chicken erythrocyte DNA, 10% glycerol, 20 nM MutY, and 1 nM DNA. The reaction mixtures were incubated at 16°C for 15 min and were electrophoresed on a 6% native polyacrylamide gel in 1xTAE buffer at 150 V for 120 min at 4°C. Binding assays with p26 or p26 D138N were carried out under the same conditions, except the protein concentration was 190 nM. For titration experiments 0.1 nM or 1 nM DNA was used in reaction mixtures that did not contain chicken erythrocyte DNA. MutY concentration ranged from 1.2 nM to 1280 nM.

Glycosylase Activity Assays

Glycosylase activity assays with MutM were performed at 37°C for 90 min in assay buffer containing 20 mM Tris-Cl, pH 7.6, 100 mM KCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 1 mM EGTA, 5 nM ^{32}P-labeled DNA, and 50 nM MutM. The reaction products were then analyzed on a 20% denaturing polyacrylamide gel. Glycosylase activity assays with MutY were performed at 37°C

for 90 min in assay buffer containing 20 mM Tris-Cl, pH 7.6, 80 mM NaCl, 1 mM DTT, 1 mM

EDTA, 1 nM ^{32}P-labeled DNA, and 100 nM MutY. The DNA was then chemically cleaved (0.1 N

NaOH) at abasic sites and the reaction products were analyzed in a 20 % denaturing gel. Glycosylase

assays with p26 and D138N p26 proteins were performed under identical conditions except the

protein concentration was 1 μM.

Glycosylase activity assays of compound lesions were performed at 37°C for 90 min in assay

buffer containing 10 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10 mM EDTA, and 50 or 140 nM

MutY, 240 or 480 nM p26, or 480 nM D138N p26. The DNA was then chemically cleaved (0.1 N

NaOH) at abasic sites and the reaction products were analyzed on a 20 % denaturing gel. For time

course experiments 1 nM ^{32}P-labeled DNA was incubated with 200 nM protein, and at various times

aliquots of the reaction mixture were removed and immediately quenched by treating with 0.1 N

NaOH. Amounts of full length and cleaved ^{32}P-labeled oligonucleotide were determined by

quantitative analysis of gels using a Molecular Dynamics PhosphorImager.

C. Results

Wild Type Cells Are More Sensitive to Cisplatin than mutM Cells

The MutM protein is a bifunctional DNA glycosylase that excises ring opened purines from

DNA (Boiteux et al., 1989; Boiteux et al., 1990; Breimer, 1984; Chetsanga et al., 1982; Chetsanga

et al., 1981; Chetsanga and Lindahl, 1979; Laval et al., 1991; O'Connor et al., 1989) and can protect

E. coli and Chinese hamster ovary cells from agents that introduce ring opened purines in DNA

(Cussac and Laval, 1996; Gill et al., 1996). To determine whether MutM could offer protection to

other agents that alkylate the N7 position of guanine, the survival of wild type and *mutM* cells after treatment with cisplatin was determined. Wild type cells were significantly more sensitive to cisplatin than *mutM* cells (Figure 3.2A). Interruption of the *uvrB* gene in these cells greatly reduced survival as previously reported (Alazard et al., 1982; Beck et al., 1985). Interestingly, in this nucleotide excision repair deficient background the differential sensitivity to cisplatin was essentially abolished; *uvrB* and *uvrB mutM* cells were equally sensitive to cisplatin (Figure 3.2B). Consequently, we propose that the different response of wild type and *mutM* cells results from modulation of the action of the UvrB protein by the MutM protein.

To determine whether MutM protein plays a role in the antitumor efficacy of platinum drugs, we measured the survival of wild type and *mutM* cells after treatment with *trans*-DDP. In contrast to cisplatin, *trans*-DDP is equally toxic to wild type and *mutM* cells (Figure 3.2C).

Purified MutM Protein Recognizes Cisplatin Intrastrand Crosslinks

Based on the difference in sensitivity to cisplatin of wild type and *mutM* cells, we hypothesized that MutM protein recognizes cisplatin adducts. Thus, we studied the interaction of purified MutM protein with cisplatin adducts. Oligonucleotides containing each of the cisplatin intrastrand crosslinks were constructed, ^{32}P-labeled, annealed to their complementary strand, and used in binding assays with MutM protein. MutM protein readily recognized all three cisplatin intrastrand crosslinks (Figure 3.3A Lanes 4, 6 and 8) under conditions that showed minimal binding to unmodified DNA (Figure 3.3A Lane 2). MutM appeared to recognize the 1,2-intrastrand crosslinks more efficiently than the 1,3-intrastrand crosslink. Thermodynamic analysis of the

binding of MutM to each of the adducts is necessary to confirm this hypothesis.

To test whether there were a direct involvement of MutM in the repair of cisplatin adducts, we looked for excision activity of the protein towards the adducts *in vitro*. No glycosylase activity of MutM protein towards any of the oligonucleotides containing the cisplatin intrastrand crosslinks was observed (Figure 3.3B lanes 6, 8, 10, 14, 16 and 18); in contrast, under these experimental conditions an AP site was efficiently cleaved by the enzyme (Figure 3.3B lane 2), confirming that the protein was active.

Since wild type and *mutM* cells were equally sensitive to *trans*-DDP, we studied the interaction of MutM protein with *trans*-DDP adducts. Plasmid DNA was globally modified with cisplatin or *trans*-DDP, ^{32}P-labeled and used in binding assays with MutM protein. The cisplatin adducts were readily recognized by the MutM protein (Figure 3.4, Lane 4); in contrast, MutM protein did not recognize *trans*-DDP adducts or unmodified DNA (Figure 3.4 Lanes 6 and 2 respectively). This result is consistent with the hypothesis that MutM recognizes the 1,2-intrastrand crosslinks formed by cisplatin. In *trans*-DDP the chloride ligands are in *trans* geometry precluding the formation of the 1,2-crosslinks, whereas the *cis* geometry in cisplatin enables their formation.

MutY Recognizes Cisplatin Adducts

In order to ascertain whether cisplatin adducts are recognized by MutY, ^{32}P-labeled duplex oligonucleotides containing each of the cisplatin intrastrand crosslinks were used in binding assays with purified MutY protein. MutY readily recognized all three intrastrand crosslinks (Figure 3.5

Lanes 4, 6 and 8). In contrast, under the same experimental conditions, little binding to unmodified DNA was observed (Figure 3.5 Lane 2).

To determine the relative binding affinities of MutY for the adducts, the oligonucleotides were incubated with increasing amounts of MutY protein. Increasing the MutY concentration in the binding reactions resulted in an increase in the amount of the shifted probe. Even though the experiment was not performed a sufficient number of times under the same experimental conditions to allow determination of the binding affinities of MutY for the adducts, it appears that MutY recognized the 1,3-d(GpTpG) adduct (Figure 3.6A) more efficiently than the 1,2-d(GpG) adduct (Figure 3.6B), which, in turn, was recognized more efficiently than the 1,2-d(ApG) adduct (Figure 3.6C).

The structures of DNA glycosylases and the NMR solution structure of DNA containing the 1,3-d(GpTpG) cisplatin adduct provide information with respect to the binding preference of MutY for the 1,3-d(GpTpG) adduct. The crystal structures of different DNA glycosylases in complex with their substrates or substrate analogues have revealed that an unpaired nucleotide is rotated out of the DNA helix of the substrate into a pocket in the enzyme surface by a process termed base flipping. The nucleotide residue has to be flipped out of the DNA helix for catalytic chemistry to ensue. Verdine and Bruner (Verdine and Bruner, 1997) propose that DNA glycosylases play an active role in extruding the base from the helix and presenting it to the active site. This process requires energy to break the hydrogen bonds of the base pair, and, therefore, this model implies that structures in which the base pairing is already disrupted might be recognized by glycosylases. The structure of the

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1,3-d(GpG) adduct reveals that the base pairing of the 5' platinated guanine and of the central thymine are disrupted, and the thymine is extruded from the minor groove (Teuben et al., 1999; van Garderen and van Houte, 1994). Since no energy would be required to break the hydrogen bonds of the central adenine and thymine base pair, the adenine residue might be easily rotated out and into the binding pocket of MutY, thereby making the interaction of MutY with the 1,3-d(GpTpG) adduct more favorable.

To examine whether MutY recognizes specifically adducts of clinically effective platinum drugs, we used plasmid DNA, globally modified with cisplatin or trans-DDP, in binding assays with MutY protein. MutY recognized both cisplatin and trans-DDP adducts (Figure 3.7A Lanes 4 and 6). This result is consistent with the observation that the 1,3-d(GpTpG) adduct is the preferred substrate for binding to MutY. trans-DDP can also form 1,3-intrastrand crosslinks that have some structural similarities to the 1,3-intrastrand crosslinks of cisplatin, such as the destacking of the central nucleotide.

To characterize the nature of the interaction of MutY with cisplatin and trans-DDP adducts, increasing amount of MutY were titrated into binding reactions with cisplatin or trans-DDP modified DNA. Binding to cisplatin adducts was observed at lower MutY concentrations than binding to trans-DDP adducts (Figure 3.7B), suggesting that MutY has a higher binding affinity for cisplatin adducts.

Glycosylase Activity Assays: *MutY Protein Can Remove Adenine Incorporated Opposite Platinated Guanines*

To determine whether MutY could excise the cisplatin adducts, duplex oligonucleotides, ^{32}P-labeled either on the adduct containing strand (Figure 3.8 Lanes 5-10), or the opposite strand (Figure 3.8 Lanes 11-16), were used in glycosylase activity assays. Each duplex was incubated with 100 nM MutY at 37°C for 90 min and reactions were terminated by addition of NaOH. Analysis of the reaction products on a denaturing polyacrylamide gel showed that MutY did not excise any of the cisplatin adducts, or any of the bases in the complementary strand. In contrast, MutY was very efficient at excising adenine from an A: G mismatch, indicating that the enzyme was active.

To probe whether the presence of a platinum adduct impairs the ability of MutY to remove adenine, we constructed a set of oligonucleotide duplexes containing an adenine opposite each of the platinated guanines of the 1,2-d(ApG), 1,2-d(GpG) or 1,3-d(GpTpG) adducts, or a guanine opposite the platinated adenine of the 1,2-d(ApG) adduct, and used them in glycosylase activity assays with purified MutY protein (Figure 3.9). Interestingly, the presence of the platinum adduct did not abolish the ability of MutY to remove adenine from any of the duplexes tested. Indeed, when adenine was incorporated opposite the platinated guanine of the 1,2-d(ApG) adduct (Figure 3.9 Lanes 11 and 12), excision of adenine was very efficient (see below). When adenine was incorporated opposite a platinated guanine of the 1,2-d(GpG) or the 1,3-d(GpTpG) adduct, the extent of cleavage by MutY was reduced compared to that of a single A: G mismatch (Figure 3.9: Compare lanes 16, 18 and 22 with lane 4), suggesting that the presence of the platinum adduct somehow hinders the ability of MutY to excise adenine. Interestingly, the amount of adenine excised when

adenine was paired with the 3' guanine of the 1,2-d(GpG) adduct was twice as much as the amount excised when adenine was paired with the 5' guanine of the 1,2-d(GpG) adduct. This result would suggest that the structure of the 3' guanine of the 1,2-d(GpG) adduct looks more like a normal base. NMR and X-ray crystallography studies have been used to study the structure of the 1,2-d(GpG) adduct (Huang et al., 1995; Takahara et al., 1995; Takahara et al., 1996; Yang et al., 1995). Interestingly, the DNA 5' to the platinated site has A like properties, whereas that to the 3' site looks more like normal B form DNA. Finally, MutY was able to excise the platinated adenine from the 1,2-d(ApG) adduct when it was paired with guanine (Figure 3.9 Lane 8), implicating MutY in the direct repair of cisplatin adducts. MutY had no activity on the G-containing strand of any of the mismatches tested (data not shown).

Interactions of p26 and Mutant D138N p26 with Cisplatin Adducts

Proteolysis of MutY yields a 26 kDa and a 13 kDa fragment (Gogos et al., 1996; Manuel et al., 1996). The large N-terminal, 26 kDa domain is called p26 and has similar substrate specificity to that of the full length protein; p26 recognizes A: G and A: 8-oxoG mismatches (Gogos et al., 1996; Li et al., 2000; Manuel and Lloyd, 1997). Furthermore, the p26 domain retains the ability to excise adenine when paired with guanine or 8-oxoG (Gogos et al., 1996; Li et al., 2000; Manuel and Lloyd, 1997; Manuel et al., 1996; Noll et al., 1999). A site directed D138N mutant p26 can still bind to DNA containing A: G and A: 8-oxoG mismatches, albeit with slightly lower affinity than wild type enzyme, but has lost the ability to remove adenine from A: G and A: 8-oxoG mismatches (Guan et al., 1998). To determine whether the p26 fragment of MutY, as well as the D138N p26 mutant protein, retain the ability to recognize cisplatin adducts, we used these proteins in binding assays

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with duplex oligonucleotides containing each of the cisplatin intrastrand crosslinks. Both p26 and

D138N p26 were able to recognize the 1,2-d(ApG) (Figure 3.10 Lanes 6 and 16), the 1,2-d(GpG)

(Figure 3.10 Lanes 8 and 18), and the 1,3-d(GpTpG) (Figure 3.10 Lanes 10 and 20) cisplatin adducts.

For comparison, the binding to A: G containing oligonucleotides was determined. Both proteins

readily recognized the mismatch (Figure 3.10 Lanes 4 and 14). In contrast, little binding to

unmodified DNA was observed (Figure 3.10 Lanes 2 and 12).

To determine whether p26 and D138N p26 retained the ability to remove adenine paired with

a platinated guanine, we used these proteins in glycosylase activity assays. Similar to intact MutY,

p26 was able to excise adenine incorporated opposite a platinated guanine as well as the platinated

adenine of the 1,2-d(ApG) adduct when paired with a guanine (Figure 3.11A Lane 14 and Figure

3.11B Lanes 5, 8, and 14). The D138N p26 mutant protein was unable to excise adenine from an

A: G mismatch (Figure 3.11A Lane 6) as previously reported (Guan et al., 1998). Furthermore, the

protein did not remove adenine incorporated opposite the platinated guanines of the 1,2-d(GpG) or

the 1,3-d(GpTpG) adducts (Figure 3.11B Lanes 6, 9, and 15). Interestingly, however, this protein

was able to remove adenine incorporated opposite the platinated guanine of the 1,2-d(ApG) adduct

(Figure 3.11A Lane 15), suggesting that this mutant protein is not catalytically dead as previously

assumed. To understand better the nature of the excision reaction, we measured the excision of

adenine from the compound lesion by p26 D138N over time. A representative gel is shown in Figure

3.12A. Quantitation of the cleaved product demonstrated that the amount of product formed

increased linearly in the first 10-15 min and then it reached a plateau at about 1% cleavage (Figure

3.12B). This result suggests that the D138N p26 mutant protein retains some catalytic activity.

Kinetics of Repair of the Compound Lesion by MutY and p26

Kinetics of adenine removal from A: G mismatches or 1,2-d(ApG/TpA) cisplatin adducts were carried out under single turnover conditions ([enzyme]>>[DNA]). The A-containing strand was ^{32}P-labeled, annealed to the complementary strand, and incubated at 37°C with 200 nM MutY. At various times (12 sec to 48 min), aliquots were removed and reactions were quenched by addition of 0.1 N NaOH. Base treatment at 70°C for 20 min effected strand scission at AP sites, generating a smaller ^{32}P-labeled DNA fragment that can be separated from the full length oligonucleotide in a denaturing polyacrylamide gel. The autoradiogram of a representative gel is shown in Figure 3.13A. Quantitation of the extent of cleavage over time demonstrated that the rate of cleavage of the A: G containing oligonucleotide was slower than the rate of cleavage of the oligonucleotide containing the 1,2-d(ApG/TpA) adduct (Figure 3.13B), suggesting that the MutY is intrinsically more reactive with the 1,2-d(ApG/TpA) adduct than with the single A: G mismatch. Similar results have been obtained when the removal of adenine opposite 8-oxoG or G was measured; the intrinsic rate of A removal from an A: 8-oxoG mismatch is faster than the rate of A removal from the corresponding A: G mismatch (Porello et al., 1998).

Work from several groups suggests that the C-terminal domain of the MutY protein plays a role in the recognition of A: 8-oxoG lesions. Removal of this domain results in lower binding affinity of the truncated protein for the A: 8-oxoG mismatch (Gogos et al., 1996; Li et al., 2000; Manuel and Lloyd, 1997). Furthermore, the truncated protein loses its catalytic preference for A: 8-oxoG over A: G (Li et al., 2000; Noll et al., 1999). To determine whether the truncated protein has a different rate for adenine removal from the 1,2-d(ApG/TpA) adduct than the full length protein,

we measured the glycosylase activity of MutY and p26 on the 1,2-d(ApG/TpA) adduct over time. A representative gel from one such experiment is shown in Figure 3.14A. Excision of A from the 1,2-d(ApG/TpA) cisplatin adduct increased linearly with time for the first 3-6 min, and then it reached a plateau at 35% cleavage. MutY and p26 displayed identical kinetic behavior towards the 1,2-d(ApG/TpA) adduct (Figure 3.14B), suggesting that the C-terminal domain of MutY does not play a role in the excision of adenine from cisplatin compound lesions.

The repair kinetics of adenine excision from an A: G mismatch by MutY or p26 were also determined. MutY was slightly more efficient than p26 at removing adenine from an A: G mismatch (Figure 3.15A). This is in contrast to Li et al. (Li et al., 2000) who reported that the rate of excision of adenine from A: G by MutY and p26 were the same, and this disrepancy may be due to the different methods used to quench the reactions. We immediately treated our samples with 0.1 N NaOH, whereas they froze their samples in a dry ice bath.

Finally, the repair kinetics of adenine excision from the compound lesion or the A: G mismatch were investigated. As seen with the full length protein, the rate of adenine excision from the compound lesion is faster than the rate of adenine excision from the A: G mismatch (Figure 3.15B).

Wild Type Cells Are Less Sensitive to Cisplatin than mutY Cells

To determine whether the ability of MutY to recognize cisplatin adducts and to excise adenine incorporated opposite platinated guanines had any biological significance, we measured the

survival of wild type and *mutY* cells after treatment with cisplatin. The *mutY* cells were more sensitive to cisplatin than wild type cells (Figure 3.16A), suggesting that the MutY protein protects cells from cisplatin toxicity. To examine whether the sensitivity of *mutY* cells was mediated by MutM, we treated *mutM* and *mutM mutY* cells with cisplatin and determined their survival. The same result was observed in this *mutM* deficient background; *mutM mutY* cells were more sensitive to cisplatin than *mutM* cells (Figure 3.16B).

MutY might facilitate cell survival through its direct interaction with cisplatin adducts, it might enhance survival because it can generate a nick opposite the site of the adduct that could serve as signal for another repair process, or it might protect cells from cisplatin toxicity because it can remove one of the adducts. To distinguish among these possibilities, plasmid DNA carrying the genes for MutY, p26, or D138N p26 was electroporated in *mutY* cells, expression of these proteins was induced by addition of 100 µM IPTG in the growth medium, and survival of the cells after treatment with cisplatin was determined. Complementation of *mutY* cells with MutY, or p26 but not with the mutant D138N p26 rescued their sensitivity to cisplatin (Figure 3.16C) suggesting that the excision ability of MutY is required to protect cells from cisplatin toxicity.

Finally, to determine whether MutY may play a role in the clinical efficacy of platinum drugs, we measured the survival of *mutY* cells after treatment with *trans*-DDP. Similar to the *mutM* strain, wild type and *mutY* cells are equally sensitive to *trans*-DDP (Figure 3.17).

MutY is Required for Cisplatin Induced Mutagenesis

Since mutagenic bypass of cisplatin adducts is required for incorporation of A opposite platinum adducts, we determined the mutagenic potency of cisplatin in wild type, *mutM*, *mutY* and *mutM mutY* cells. Increasing the concentration of cisplatin used to treat the cells resulted in an increase in the number of rifampicin resistant cells in wild type and *mutM* strains (Figure 3.18A). Only at the higher cisplatin concentrations used did we observe cisplatin induced mutagenesis in *mutY* cells, and the effect was much less pronounced than in wild type and *mutM* cells (Figure 3.18A). In the *mutM mutY* strain, with the cisplatin doses used, no induction in mutagenesis was observed (Figure 3.18A). Note that the spontaneous level of rifampicin resistant cells in *mutM*, *mutY* or *mutM mutY* cells was significantly increased over the background observed in wild type cells (Table 3.2) as previously reported (Cabrera et al., 1988; Michaels et al., 1992; Nghiem et al., 1988).

Strain	Fold Increase in Mutation Frequency
CC104	1
CC104 mutM	2.3
CC104 mutY	8.2
CC104 mutM mutY	128.6

Table 3.2: Fold increase in the mutation frequency

Since the strains used in this experiment are differentially sensitive to cisplatin, we determined the mutagenic potency of cisplatin at equitoxic doses. At a dose that results in a 70% survival of the cells, the fold increase in cisplatin mutagenesis in wild type and *mutM* strains was not significantly different (Figure 3.18B). In contrast, *mutY* and *mutM mutY* were extremely resistant

to cisplatin mutagenesis (Figure 3.18B).

It is noteworthy that *mutM mutY* strains are not generally resistant to chemical induced mutagenesis. For example, t-butyl hydroperoxide is more efficient at inducing Trp^+ revertants in *uvrA umuD,C mutM mutY* cells than in *uvrA umuD,C* cells (Urios and Blanco, 1996). Moreover, t-butyl hydroperoxide and cumene hydroperoxide can increase the number of Trp^+ revertants in both wild type and *mutY* cells (Urios et al., 1995). Finally, benzo[a]pyrene induced low levels of Trp^+ revertants in wild type and *mutM* cells whereas it was very efficient at inducing Trp^+ revertants in *mutY* cells (Urios and Blanco, 1996).

Cisplatin mutagenesis in wild type cells may be mediated by the binding of MutY to cisplatin adducts and shielding of these adducts from repair. Alternatively, MutY might increase the mutagenicity of cisplatin by generating a nick opposite the lesion that can serve as a signal for another mutagenic process. To distinguish between these possibilities, we introduced into *mutY* cells plasmid DNA expressing MutY, p26, or D138N p26 proteins and examined the effect of expression of these proteins on cisplatin induced mutagenesis. Under non-inducing conditions the response of cells expressing these proteins was the same as that of the *mutY* cells; basal expression of MutY, p26, or D138N p26 did not increase the mutagenic potency of cisplatin in *mutY* strains (Figure 3.19A). Induction of expression of the proteins by 100 μM IPTG however, resulted in partial rescue of wild type response (Figure 3.19B).

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D. Discussion

MutM May Mediate Cisplatin Toxicity by Shielding Adducts from Repair

In this report we demonstrated that the MutM glycosylase recognized the intrastrand crosslinks formed by cisplatin but had no excision activity on these adducts. Furthermore, *in vivo* experiments with wild type and *mutM* cells demonstrated that the presence of the MutM protein sensitized cells to cisplatin, and this effect of MutM was essentially abolished in a nucleotide excision repair deficient background, suggesting that MutM somehow modulates the activity of nucleotide excision repair proteins on cisplatin adducts. Analogous observations have been made with the HMG family of proteins. HMG box proteins have been shown to interact with clinically effective platinum drugs (Pil and Lippard, 1992; Toney et al., 1989), and *in vitro* repair assays have shown that the presence of HMG box proteins in repair reactions inhibit the repair of cisplatin adducts by the nucleotide excision repair machinery (Huang et al., 1994; Trimmer et al., 1998; Zamble et al., 1996), leading to the proposal of a model where HMG box proteins mediate cisplatin toxicity by binding to cisplatin adducts and shielding them from repair. Support for this model comes from studies with *Saccharomyces cerevisiae* cells. Cells deficient in Ixr1, a *S. cerevisiae* HMG box protein, are 2-6 fold more resistant to cisplatin than wild type cells (Brown et al., 1993; McA'Nulty and Lippard, 1996), and this differential sensitivity is not observed in the absence of nucleotide excision repair (McA'Nulty and Lippard, 1996). Consequently, we propose that similar to the HMG box proteins, MutM binds to cisplatin adducts and physically blocks their recognition by other repair proteins. The adducts persist in DNA, thereby potentiating the toxicity of cisplatin (Figure 3.20). In a *mutM* cell there is no repair shielding, the adducts get repair faster, and cisplatin toxicity is diminished.

MutY Protects E. coli Cells from Cisplatin Toxicity by Facilitating the Repair of Cisplatin Adducts

The majority of mutations caused by cisplatin adducts are G to T at either the 5' guanine (Bradley et al., 1993; Yarema et al., 1995) or the 3' guanine (Lemaire et al., 1991) of the 1,2-d(GpG) adduct, and A to T (Burnouf et al., 1990; Yarema et al., 1995) or A to C (Burnouf et al., 1990) at the 5' adenine of the 1,2-d(ApG) adduct, indicating that the 1,2-intrastrand crosslinks are frequently mispaired with adenine *in vivo*.

MutY is an adenine glycosylase that repairs A: G and A: 8-oxoG mismatches to C: G (Au et al., 1989; Michaels et al., 1992; Michaels et al., 1992; Tsai-Wu et al., 1992). MutY can also remove adenine from A: C and A: 8-oxoA mismatches (Michaels et al., 1992; Tsai-Wu et al., 1992) and guanine from G: 8-oxoG mismatches (Zhang et al., 1998). In this investigation we demonstrated that MutY can recognize cisplatin adducts and can excise adenine incorporated opposite a platinated guanine. Furthermore, MutY was able to excise the platinated adenine of the 1,2-d(ApG) adduct when paired with guanine, suggesting that MutY might be directly involved in the repair of cisplatin adducts *in vivo*. The interaction between MutY and cisplatin adducts appears to be very important since *mutY* cells were significantly more sensitive to cisplatin. Moreover, expression of MutY or p26, but not D138N p26, in *mutY* cells rescued their sensitivity to cisplatin, indicating that the catalytic activity of MutY is important for survival after treatment with cisplatin.

We propose that MutY protects cells from cisplatin toxicity by facilitating the repair and/or increasing the tolerance of the cell for cisplatin adducts. First, MutY improves survival by initiating the removal of the 1,2-d(ApG) adduct (Figure 3.21). There have been several studies that correlate

the rate of repair of the 1,2-d(ApG) adduct with cellular resistance to cisplatin (Bedford et al., 1988; Dempke et al., 1992; Fichtinger-Schepman et al., 1995; Hill et al., 1994a; Hill et al., 1994b; Shellard et al., 1993), suggesting that the 1,2-d(ApG) crosslink is a key contributor to the toxicity of cisplatin. In a *mutY* cell the excision of the 1,2-d(ApG) adduct would be less efficient, the adduct would persist in DNA, and cisplatin toxicity would be increased. Secondly, the nick generated opposite a cisplatin adduct after the action of MutY (and an AP endonuclease) could serve as a signal for another repair or tolerance pathway (Figure 3.21). In support of this model is the fact that strand breaks are signals for recombination, a known system for cisplatin damage tolerance (Zdraveski et al., 2000). Both mechanisms result in a more efficient processing of cisplatin adducts and in an increased survival after cisplatin treatment. A similar model has been proposed for the bacterial photolyase enzyme. *In vitro* studies have shown that this enzyme, in addition to being involved in the repair of thymine photodimers, recognizes the 1,2-d(GpG) cisplatin adduct and recruits nucleotide excision repair (Ozer et al., 1995).

MutY is Required for Cisplatin Mutagenesis

Cisplatin mutagenesis is dependent on LexA and RecA activities, suggesting that SOS processing of cisplatin adducts is required for mutagenesis (Konishi et al., 1981). Cisplatin adducts are blocks to DNA replication, and SOS induced expression of low fidelity polymerases is required for bypass of the adducts. Accordingly, cisplatin is not mutagenic in *umuD, C* cells (Fram et al., 1985). Moreover, induction of SOS increases the mutation frequency of cisplatin (Bradley et al., 1993; Yarema et al., 1995). Interestingly, cisplatin mutagenesis is particularly dependent on the recombinational capability of RecA (Jarosik and Beck, 1984). In this report we demonstrated that

MutY activity was also required for cisplatin mutagenesis suggesting that some repair proteins might protect cells from cisplatin toxicity at the expense of the integrity of the DNA genome. A similar result has been observed with nucleotide excision repair deficient cells. In general, chemical and physical agents producing DNA adducts that are substrates for the nucleotide excision repair machinery are very toxic in nucleotide excision repair deficient cells. The same repair deficient cells are almost invariably much more susceptible to the mutagenic effects of the treatment. Cisplatin appears to be an exception to this rule. Several studies have shown that mutation induction by cisplatin in *uvrA* or *uvrB*, but not *uvr*C, cells is greatly diminished. Some insight on the mechanism by which nucleotide excision repair proteins enhance cisplatin mutagenesis comes from studies with the mammalian nucleotide excision repair system. Compound cisplatin adducts (i.e. cisplatin adducts in which one of the platinated bases is mispaired) are more efficiently excised by nucleotide excision repair than the simple cisplatin adducts, and this excision occurs primarily on the adduct containing strand (Moggs et al., 1997). Efficient excision of an adduct within a compound lesion and resynthesis across the gap opposite the formerly mispaired base would lead to fixation of a mutation.

In the present work we showed that expression of MutY, p26 and D138N p26 in *mutY* cells partially restored the ability of cisplatin to cause mutations, implying that the catalytic activity of MutY on cisplatin adducts is not required for induction of mutagenesis and that adduct recognition is sufficient for mutagenesis. Alternatively, MutY, p26 and D138N p26 may use two distinct mechanisms to increase the mutagenic potency of cisplatin. In the absence of MutY or p26, recognition of cisplatin adducts by nucleotide excision repair proteins is less efficient (see above).

Since UvrA and UvrB are required for the mutagenic processing of cisplatin adducts, this delay might be sufficiently long to cause cell death, or to allow a different, non-mutagenic mechanism of repair or damage tolerance to take place (Figure 3.22A). Moreover, removal of the 1,2-d(ApG) adduct when the 5' adenine is mispaired with guanine followed by resynthesis would result in an A to C mutation (Figure 3.22A). Accordingly, the 1,2-(ApG) frequently gives rise to A to C mutations (Burnouf et al., 1990). Additionally, cisplatin adducts may "hijack" MutY away from A: G and A: 8-oxoG mismatches. The mismatches then would persist in DNA, and after another round of replication the cells would have acquired a mutation. As the cisplatin concentration used to treat the cells increases, more MutY is being "hijacked", and, therefore, the ability of the cell to repair A: G and A: 8-oxoG mismatches is reduced (Figure 3.22B). In *mutY* cells where there is no MutY protein present, cisplatin adducts do not affect mutagenesis.

We propose that a different mechanism is responsible for the rescue of cisplatin induced mutagenesis of *mutY* cells expressing D138N p26. During DNA replication a guanine might be misincorporated opposite an adenine. In a normal cell, MutS and MutY compete for binding to the mismatch. If mismatch repair takes place, the guanine is removed from the mismatch, the A: T base pair is restored, and mutagenesis is prevented. If, however, MutY reaches the mismatch first, the adenine is removed thereby promoting mutagenesis (Figure 3.23A). *In vivo* experiments have demonstrated that when both pathways can function, the *mutY* pathway dominates (Au et al., 1988). Therefore, in a normal cell this competition would contribute a certain amount to the overall mutation frequency. In a *mutY* cell overexpressing D138N p26, there is a lot more D138N p26 than MutS; therefore, D138N p26 has a better chance than MutS of reaching the A: G mismatch, and

binding of this protein to the mismatch blocks access to MutS. Even a short delay in MutS recognition of the lesion is important, since *dam* methylation takes place after replication, the strand discrimination signal is lost, and mismatch repair can no longer repair the mismatch. Consequently, in the next round of replication the mutation is fixed. In cisplatin treated cells, MutS binds to cisplatin adducts, and, therefore, the effective MutS concentration available to repair A: G mismatches is reduced and mutagenesis is increased (Figure 3.23B). Consistent with this model are studies measuring the effects of overexpression of dam methylase. It was demonstrated that an increase in the levels of dam methylase results in an increase in mutation frequency (Herman and Modrich, 1981; Marinus et al., 1984). Since the rate of methylation is limited by the amount of dam methylase present inside a cell (Szyf et al., 1984), an increase in that amount would cause a faster methylation of GATC sites and therefore a decrease in the temporal window during which the daughter strand can be distinguished from the parental strand and would thus reduce postreplication mismatch correction and increase the mutation frequency.

Recognition by MutM and MutY Might Determine Clinical Efficacy of Platinum Drugs

Finally, the effect of the MutM and MutY proteins on *trans*-DDP toxicity was measured. In contrast to cisplatin, *mutY* and *mutM* cells were as sensitive to *trans*-DDP as wild type cells, suggesting that these proteins do not recognize the *trans*-DDP adducts. Binding assays confirmed that MutM recognized specifically the adducts of the clinically effective cisplatin drug. Binding studies with MutY protein demonstrated that even though the protein recognized both cisplatin and *trans*-DDP adducts, it had higher affinity for the adducts of the clinically effective cisplatin. Cisplatin intrastrand crosslinks unwind the DNA helix by 13° to 23° and bend it by 33° in the

direction of the major groove. In contrast, *trans*-DDP adducts unwind the DNA helix by 9° and allow bending in more than one direction. These observations suggest that the structural alterations induced in DNA after binding of the platinum drugs may be important determinants for MutM and MutY recognition. Further binding studies with DNA modified with other platinum drugs are required to see whether there is a correlation between adduct recognition by MutM and MutY, sensitivity of *mutM* and *mutY* cells to the drug, and clinical efficacy.

E. Future Work

Kinetic and Thermodynamic Analysis of MutY Interactions with Cisplatin Adducts

Thermodynamic analysis of MutM and MutY binding to each of the cisplatin adducts would test the models presented in the results section of this chapter for the mode of binding to cisplatin adducts, and it would provide information regarding the mechanism of recognition of the adducts by MutM and MutY. Moreover, the binding affinity for cisplatin and *trans*-DDP adducts, as well as the binding affinity for adducts formed by other platinum drugs, would test whether there is a correlation between adduct recognition by MutM and MutY and clinical efficacy.

Porello et al. (Porello et al., 1998) demonstrated that monitoring the adenine excision from A: G or A: 8-oxoG yields different results at different time points. Analysis of the reaction products after a short period of time indicates that A: 8-oxoG substrates are more reactive than A: G substrates, whereas at longer times more product is observed with the A: G substrate. MutY can have multiple catalytic turnovers with the A: G substrate, while with A: 8-oxoG it remains bound to product; hence it is unable to process any additional substrate beyond the first turnover (Porello

et al., 1998). These results indicate that under these conditions standard Michaelis-Menten kinetic parameters K_{cat} and K_M cannot be determined for the A: 8-oxoG substrates. In contrast to A: 8-oxoG, steady state kinetics of the reaction with A: G can be obtained. It would be interesting to evaluate whether 1,2-d(ApG/TpA) behaves like A: 8-oxoG or like A: G and, if possible, determine the K_{cat} and K_M parameters for the cisplatin compound lesions. We showed that the intrinsic rate for adenine excision from the 1,2-d(ApG/TpA) adduct is greater than that from an A: G mismatch. Moreover, the intrinsic rate for adenine excision from A: 8-oxoG is greater than that from the A: G mismatch; therefore, it would be interesting to compare the rates of adenine excision from 1,2-d(ApG/TpA) and A: 8-oxoG.

Determine the Role of MutM and MutY in Excision Repair of Cisplatin Adducts

We have proposed that MutM protein shields cisplatin adducts from repair. This model is easily testable *in vitro*. Excision repair of cisplatin adducts by purified UvrA, UvrB, and UvrC proteins in the presence of MutM could be compared to excision repair in the absence of MutM. Moreover, this system could be used to test whether MutY facilitates nucleotide excision repair of the adducts. DNA substrates containing the compound cisplatin adducts could be incubated with MutY prior to incubation with the Uvr(A)BC excinuclease to allow reaction to take place. More efficient repair of cisplatin adducts in the presence of MutY would be consistent with our model.

Determine the Role of MutY in Cisplatin Mutagenesis

We have proposed two non mutually exclusive models for the requirement of MutY in cisplatin mutagenesis. In the first, MutY interacts directly with the adducts and initiates a mutagenic

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process, whereas in the second, cisplatin adducts promote mutagenesis in the cell by "hijacking"

MutY away from A: 8-oxoG and A: G mismatches. To distinguish between these models and to

determine their relative importance, the following experiments are proposed.

1. Construct site specifically modified genomes with each of the cisplatin adducts and

determine the mutagenicity of each of the adducts in wild type, *mutY*, *mutM,* and *mutM mutY* cells.

If MutY is directly responsible for the mutagenic processing of cisplatin adducts, then the mutation

frequency of the adducts in wild type cells would be higher than in *mutY* cells. If, however, cisplatin

increases mutagenesis through an indirect mechanism, such as the "hijacking" of MutY from A: G

or A: 8-oxoG mismatches, then the mutation frequency of the adducts will be the same in these two

cell strains.

2. Electroporate plasmid DNA, damaged or not with cisplatin, along with plasmid DNA

containing a single 8-oxoG in wild type, *mutY*, *mutM*, and *mutM mutY* cells, and measure 8-oxoG

mutagenesis. If MutY gets "hijacked" by cisplatin adducts, there should be an increase in the

mutation frequency of wild type cells that contain plasmid DNA damaged with cisplatin compared

to wild type cells that contain unmodified DNA. Moreover, the presence of cisplatin adducts should

not affect the mutation frequency in *mutY* cells.

3. If cisplatin adducts titrate MutY away from A: 8-oxoG mismatches, then cells treated with

cisplatin will have a *mutY* mutator phenotype. Interestingly, overexpression of MutM rescues the

mutator phenotype of *mutY* cells (Michaels et al., 1992), therefore we would expect that it can also

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decrease cisplatin induced mutagenesis.

4. The occurrence of SOS independent mutations after treatment with alkyl hydroperoxides can be easily detected in strains lacking mutagenesis proteins if they are deficient in *mutY* (Urios et al., 1995; Urios and Blanco, 1996), suggesting that alkyl hydroperoxides generate 8-oxoG lesions in DNA. To test if cisplatin adducts increase mutagenesis in a cell by "hijacking" MutY away from its natural substrates, we would test whether cisplatin and agents that introduce 8-oxoG in DNA have a synergistic effect in mutation induction. For that purpose, wild type and *mutY* cells would be challenged with cisplatin alone, alkyl hydroperoxides alone, or both agents in combination, and mutation frequencies will be determined. If "hijacking" takes place, then we'd expect to see a synergistic effect in wild type cells and an additive effect in *mutY* cells. If, however, we do not observe any synergy in wild type cells, or if we see a synergistic effect in both wild type and mutY cells, then hijacking doesn't take place *in vivo*.

Test Model for D138N p26 "Rescue" of Cisplatin Mutagenesis

We've proposed that D138N p26 increases the mutagenesis of cisplatin adducts by shielding the repair of A: G mismatches and that cisplatin adducts "hijack" MutS away from A: G, thereby reducing the MutS concentration available to repair A: G mismatches. To test this hypothesis the mutagenic potency of cisplatin should be tested in a *mutY mutS* strain in the presence or absence of D138N p26 expression. In this strain there is no MutS to be "hijacked" by cisplatin adducts, therefore, there should not be any difference in cisplatin induced mutagenesis between cells that express D138N p26 and cells that do not express this protein.

Role of Human Homologue in Cellular Resistance to Cisplatin

The human homologue of the *mutY* gene (*hMYH*) has been cloned and sequenced (Slupska et al., 1996). hMYH is a 59 kDa protein that shows 41% identity to the *E. coli* MutY protein. Moreover, hMYH can remove adenine from A: 8-oxoG and A: G mismatches and after prolonged incubation it can remove guanine from G: 8-oxoG mismatches (McGoldrick et al., 1995; Slupska et al., 1999). Finally, expression of *hMYH* in *mutY* cells rescues their mutator phenotype (Slupska et al., 1999). These results suggest that MutY and hMYH have the same substrate specificity, and we propose that the hMYH protein protects human cells from cisplatin toxicity. In the absence of human cells that only differ in *hMYH* status, this hypothesis could be tested in *E. coli*. Plasmid DNA expressing *hMYH* could be electroporated into *mutY* cells, and survival of these cells after treatment with cisplatin could be compared with survival of *mutY* cells. Cisplatin toxicity studies could also be performed with fibroblasts from *mMYH*$^{+/+}$ and *mMYH*$^{-/-}$ mice. Finally, a detailed thermodynamic and kinetic analysis of the interactions of hMYH protein with cisplatin adducts could be carried out.

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1,2-d(ApG)	5′-CCTCTCCTT**AG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAATCAGAAGAGGAGAGG-5′
1,2-d(GpG)	5′-CCTCTCCTT**GG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAACCAGAAGAGGAGAGG-5′
1,3-d(GpTpG)	5′-CCTCTCCTT**GTG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAACACAGAAGAGGAGAGG-5′
1,2-d(ApG/GpC)	5′-CCTCTCCTT**AG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAAGCAGAAGAGGAGAGG-5′
1,2-d(ApG/TpA)	5′-CCTCTCCTT**AG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAATAAGAAGAGGAGAGG-5′
1,2-d(GpG/ApC)	5′-CCTCTCCTT**GG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAAACAGAAGAGGAGAGG-5′
1,2-d(GpG/CpA)	5′-CCTCTCCTT**GG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAACAAGAAGAGGAGAGG-5′
,3-d(GpTpG/ApApC)	5′-CCTCTCCTT**GTG**TCTTCTCCTCTCC-3′ 3′-GGAGAGGAAAACAGAAGAGGAGAGG-5′

Figure 3.1: Sequences of the DNA duplexes used in the binding and glycosylase experiments. The pyrimidine rich strand is designated as the top strand, and the complementary strand is designated as the bottom strand. The bases involved in adduct formation are located in the top strand and are indicated in bold. The strand containing the adenine of the A: G mismatch is designated as the A-containing strand and the strand containing the guanine of the A: G mismatch is designated as the G-containing strand.
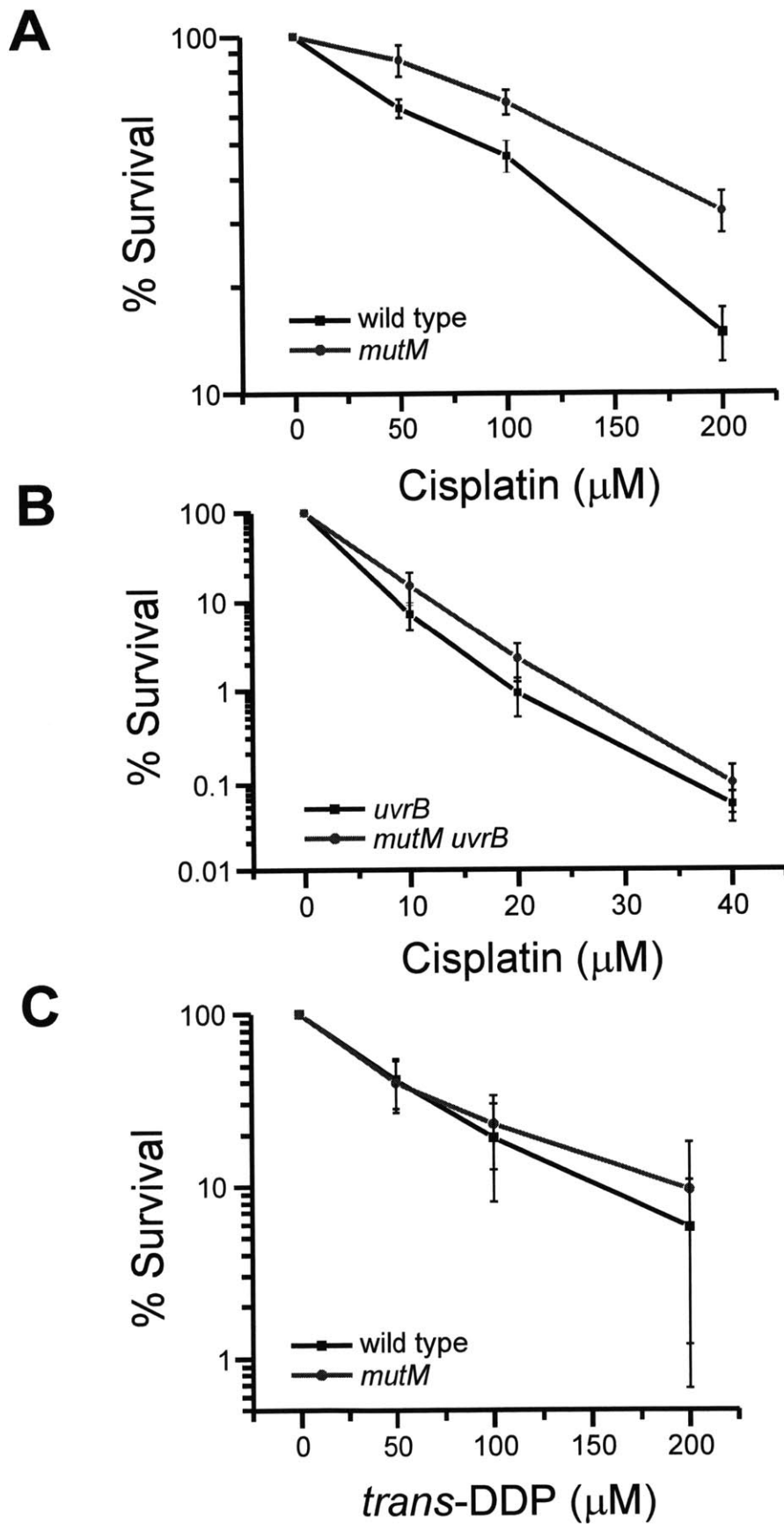
Figure 3.2: **(A)** Survival of wild type and *mutM* cells after treatment with cisplatin. **(B)** Survival of *uvrB* and *mutM uvrB* cells after treatment with cisplatin. **(C)** Survival of wild type and *mutM* cells after treatment with *trans*-DDP. The data in these graphs are the average of at least four experiments, and the error bars represent the standard error of the mean.
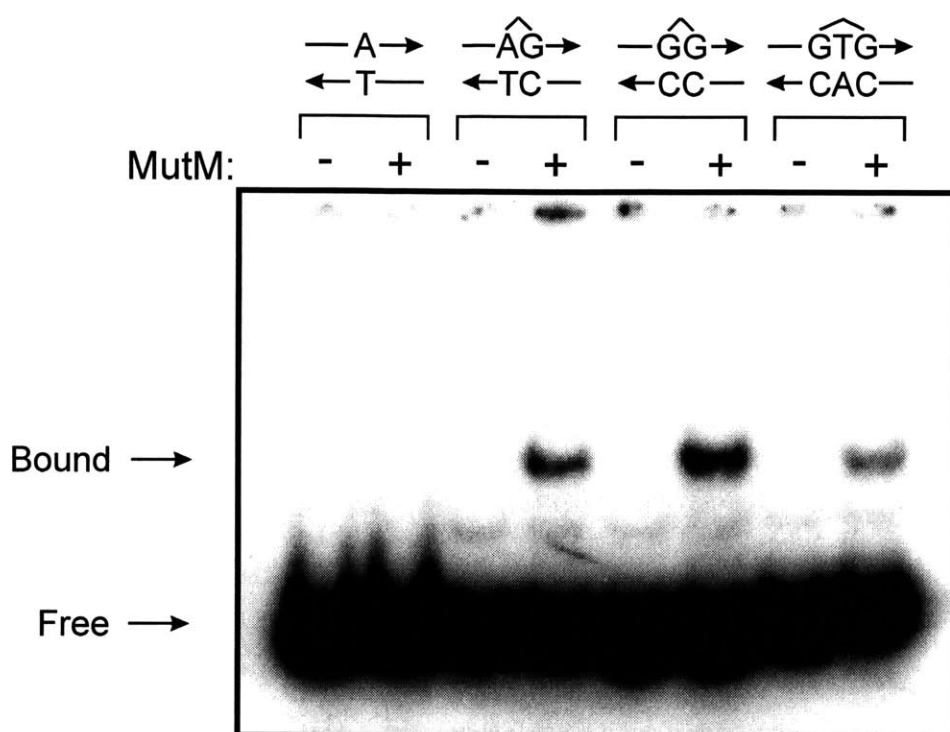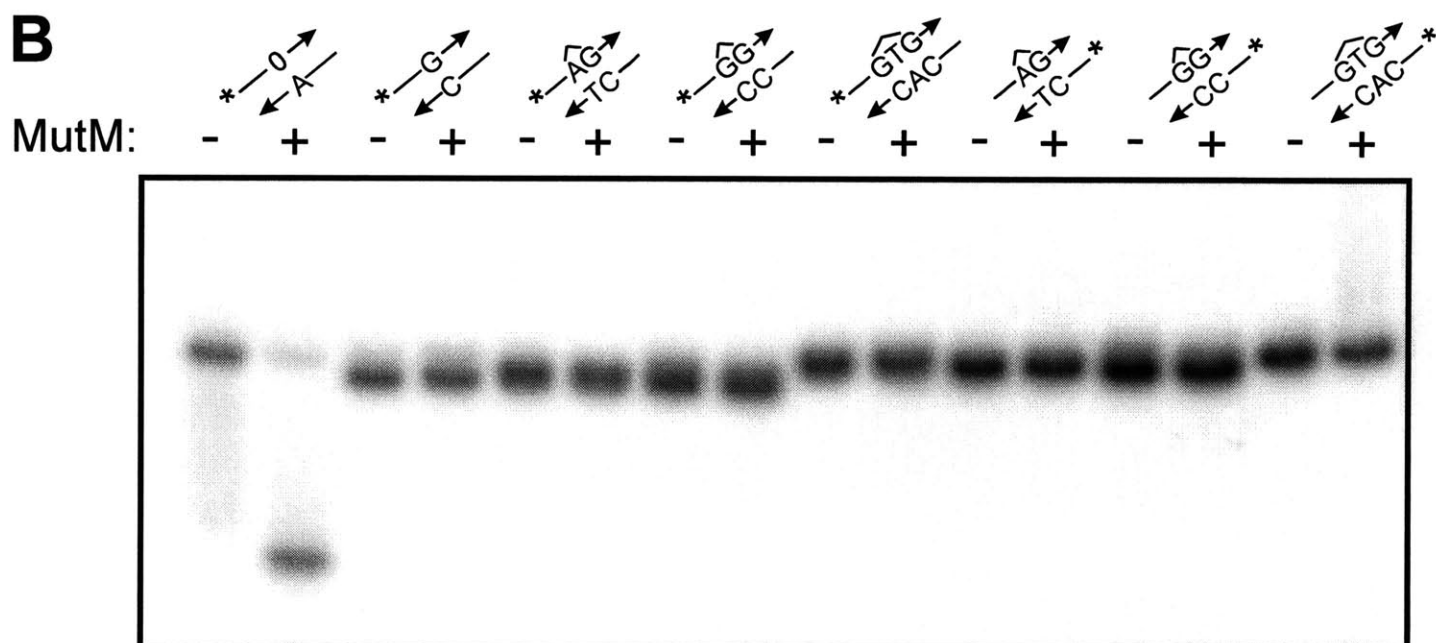
Figure 3.3: MutM recognizes the cisplatin intrastrand crosslinks. **(A)** Gel mobility shift assay of MutM with various oligonucleotide duplexes. The ^{32}P-labeled oligonucleotides containing a single 1,2-d(ApG) (Lanes 3 and 4), 1,2-d(GpG) (Lanes 5 and 6), or 1,3-d(GpTpG) (Lanes 7 and 8) were incubated in the presence (+) or absence (-) of 50 nM purified MutM. Unmodified DNA (Lanes 1 and 2) was used as a control. **(B)** Enzymatic processing of an AP site or cisplatin adducts by MutM. 100 nM MutM were incubated with an oligonucleotide containing an AP site (Lanes 1 and 2), unmodified oligonucleotide (Lanes 3, and 4), 1,2-d(ApG) oligonucleotide (Lanes 5, 6, 11, and 12), 1,2-d(GpG) oligonucleotide (Lanes 7, 8, 13, and 14), or 1,3-d(GpTpG) oligonucleotide (Lanes 9, 10, 15, and 16), ^{32}P-labeled in the top (lanes 1-10) or in the bottom strand (Lanes 11-16).
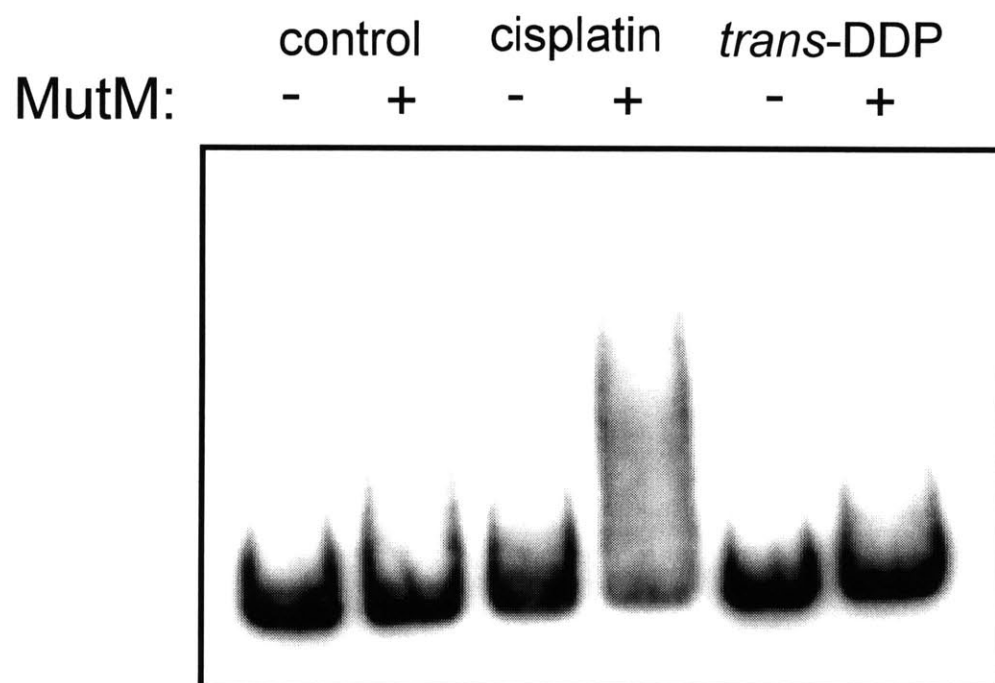
Figure 3.4: Selectivity of MutM for DNA modified with clinically effective platinum drugs. Unmodified plasmid DNA (Lanes 1 and 2) or plasmid DNA globally modified with cisplatin (Lanes 3 and 4) or *trans*-DDP (Lanes 5 and 6) was incubated in the presence (+) or absence (-) of 320 nM MutM. These binding reactions also contained 1 ng salmon sperm DNA.

Figure 3.5: Gel mobility shift assay of MutY with oligonucleotide duplexes containing cisplatin adducts. The ^{32}P-labeled oligonucleotides containing a single 1,2-d(ApG) (lanes 3 and 4), 1,2-d(GpG) (lanes 5 and 6), or 1,3-d(GpTpG) (lanes 7 and 8) were incubated in the presence (+) or absence (-) of 50 nM purified MutY. Unmodified DNA (lanes 1 and 2) was used as a control.
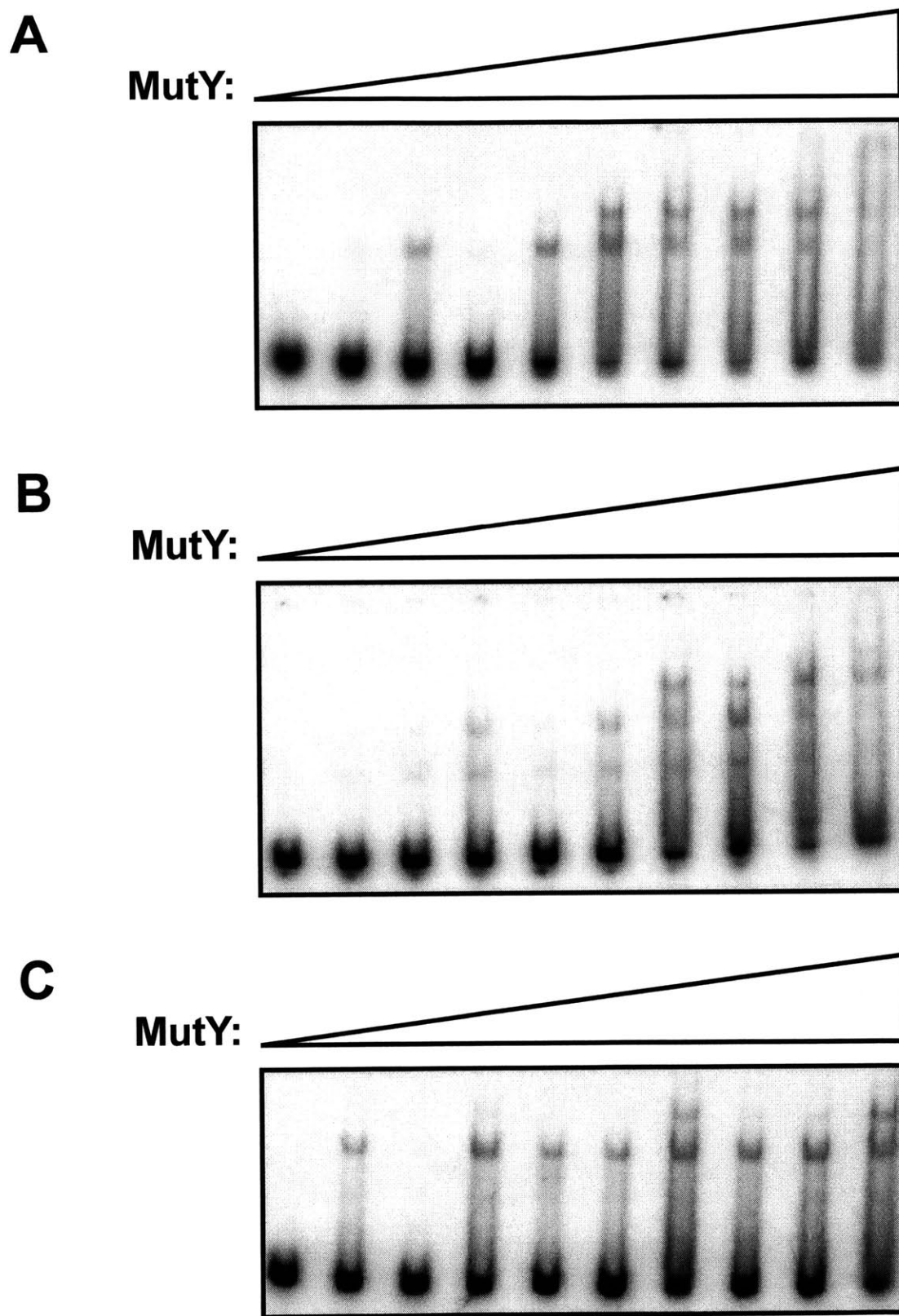
Figure 3.6: 0.2 nM of **(A)** 1,3-d(GpTpG), **(B)** 1,2-d(GpG), or **(C)** 1,2-d(ApG) ^{32}P-labeled oligonucleotides were incubated with increasing concentrations of MutY. The concentrations of MutY were 0, 5, 10, 20, 40, 80, 160, 320, 640, and 1280 nM.
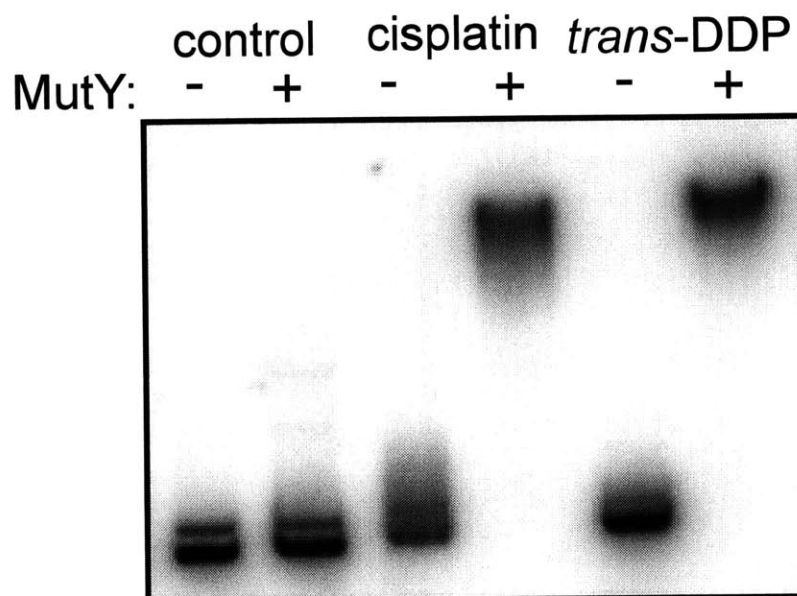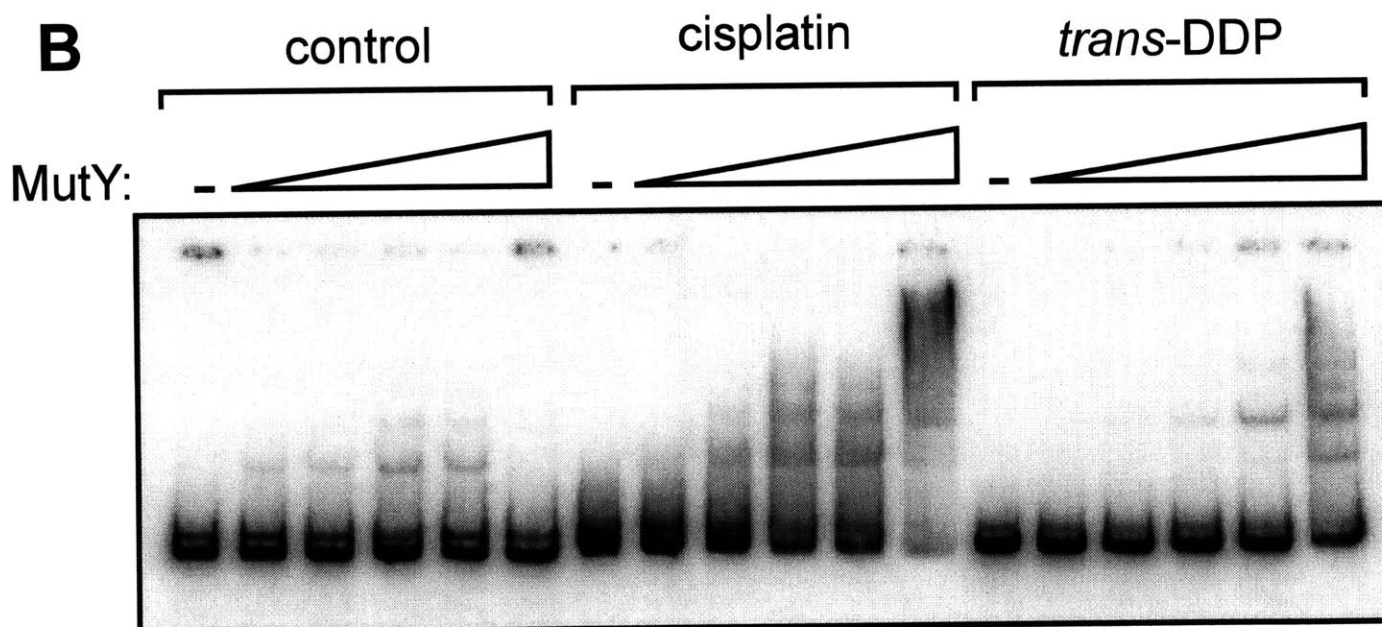
Figure 3.7: Interactions of MutY with cisplatin and *trans*-DDP adducts. **(A)** Unmodified plasmid DNA (Lanes 1 and 2), plasmid DNA globally modified with cisplatin (Lanes 3 and 4), or *trans*-DDP modified plasmid DNA (Lanes 5 and 6) was incubated in the presence (+) or absence (-) of MutY. **(B)** Unmodified plasmid DNA (Lanes 1-6), cisplatin modified DNA (Lanes 7-12), or *trans*-DDP modified DNA (Lanes 13-18) was incubated with 0, 14, 28, 42, 56 or 70 nM MutY.
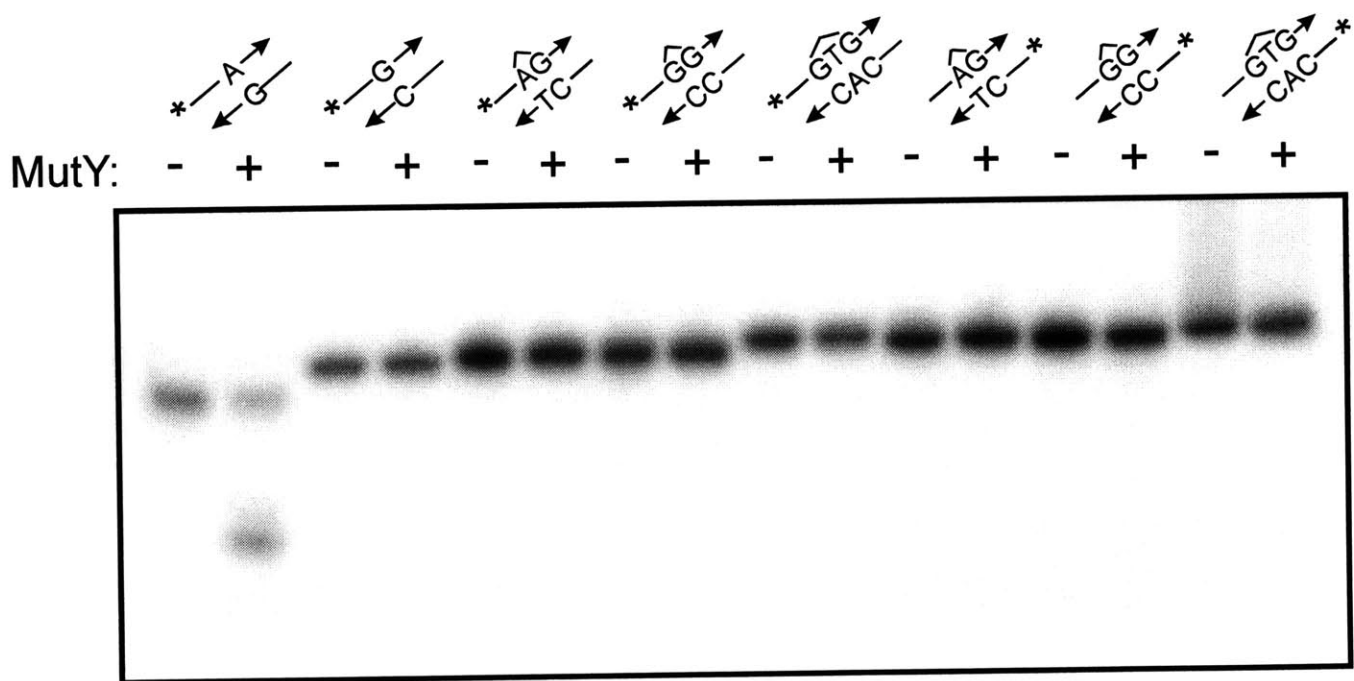
Figure 3.8: Enzymatic processing of an A: G mismatch or cisplatin adducts by MutY. 100 nM MutY were incubated with an oligonucleotide containing an A: G mismatch (Lanes 1 and 2), unmodified DNA (Lanes 3, and 4), 1,2-d(ApG) oligonucleotide (Lanes 5, 6, 11, and 12), 1,2-d(GpG) oligonucleotide (Lanes 7, 8, 13, and 14), or 1,3-d(GpTpG) oligonucleotide (Lanes 9, 10, 15, and 16), ^{32}P-labeled in the top (Lanes 1-10) or in the bottom strand (Lanes 11-16). AP sites were cleaved by treatment with 0.1 N NaOH at 70°C for 20 min.
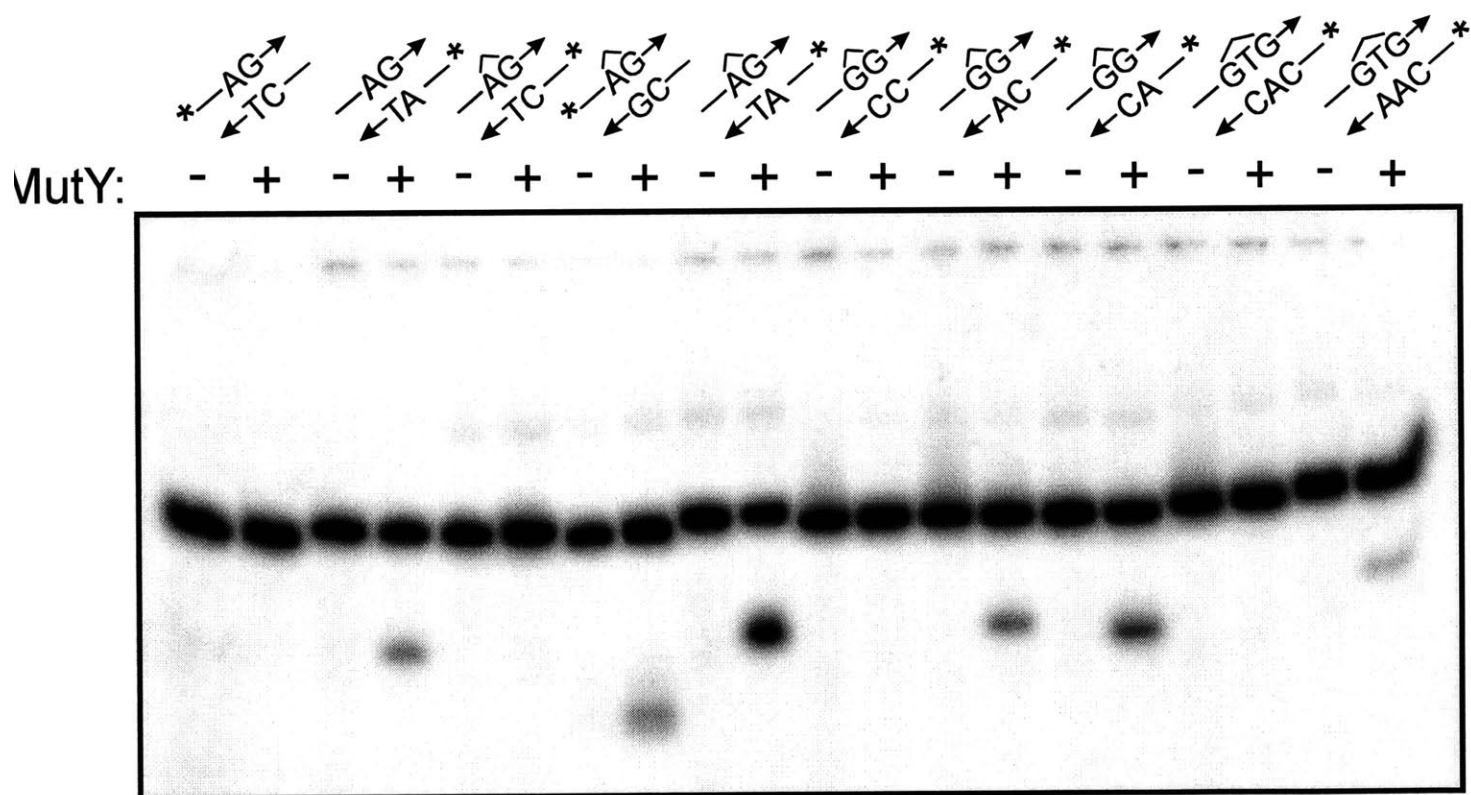
Figure 3.9: Enzymatic processing of cisplatin compound lesions by MutY. Unmodified DNA (Lanes 1 and 2), A:G mismatch containing oligonucleotide (Lanes 3 and 4), 1,2-d(ApG) oligonucleotide (Lanes 5 and 6), 1,2-d(ApG/GpC) oligonucleotide (Lanes 7 and 8), 1,2-d(ApG/TpA) oligonucleotide (Lanes 9 and 10), 1,2-d(GpG) oligonucleotide (Lanes 11 and 12), 1,2-d(GpG/ApC) oligonucleotide (Lanes 13 and 14), 1,2-d(GpG/CpA) oligonucleotide (Lanes 15 and 16), 1,3-d(GpTpG) oligonucleotide (Lanes 17 and 18), or 1,3-d(GpTpG/ApApC) oligonucleotide (Lanes 19 and 20) were incubated at 37°C for 90 min in the presence or absence of 50 nM MutY. AP sites were cleaved by treatment with 0.1 N NaOH at 70°C for 20 min. The asterisk (*) denotes the ^{32}P labeled strand.
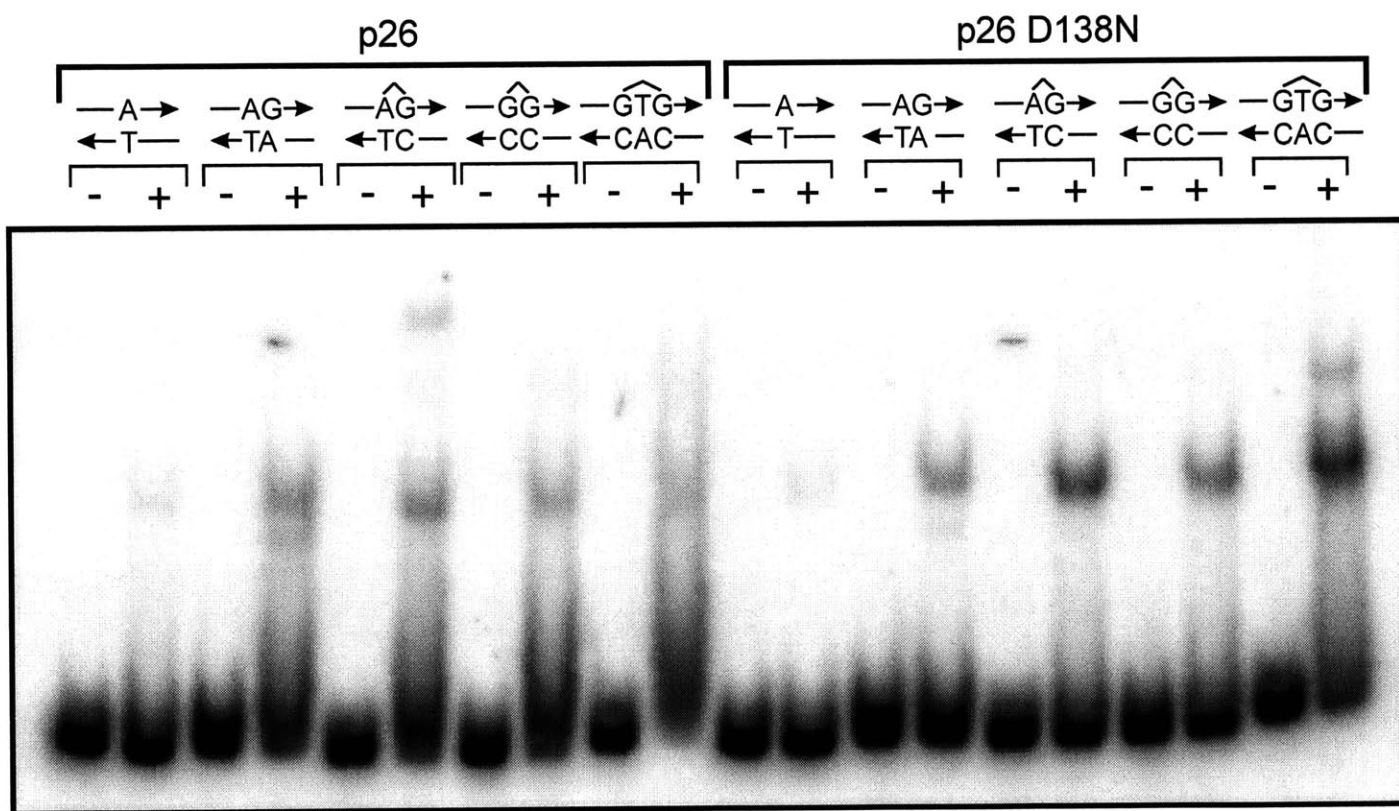
Figure 3.10: Interactions of cisplatin adducts with the catalytically active domain of MutY, p26, and the D138N p26 mutant. Unmodified DNA (Lanes 1, 2, 11 and 12), A: G mismatch containing oligonucleotide (Lanes 3, 4, 13 and 14), 1,2-d(ApG) oligonucleotide (Lanes 5, 6, 15, and 16), 1,2-d(GpG) oligonucleotide (Lanes 7, 8, 17, and 18), or 1,3-d(GpTpG) oligonucleotide (Lanes 9, 10, 19, and 20) were incubated in the presence (+) or absence (-) of 190 nM p26 (Lanes 1-10), or 190 nM D138N p26 (Lanes 11-20).
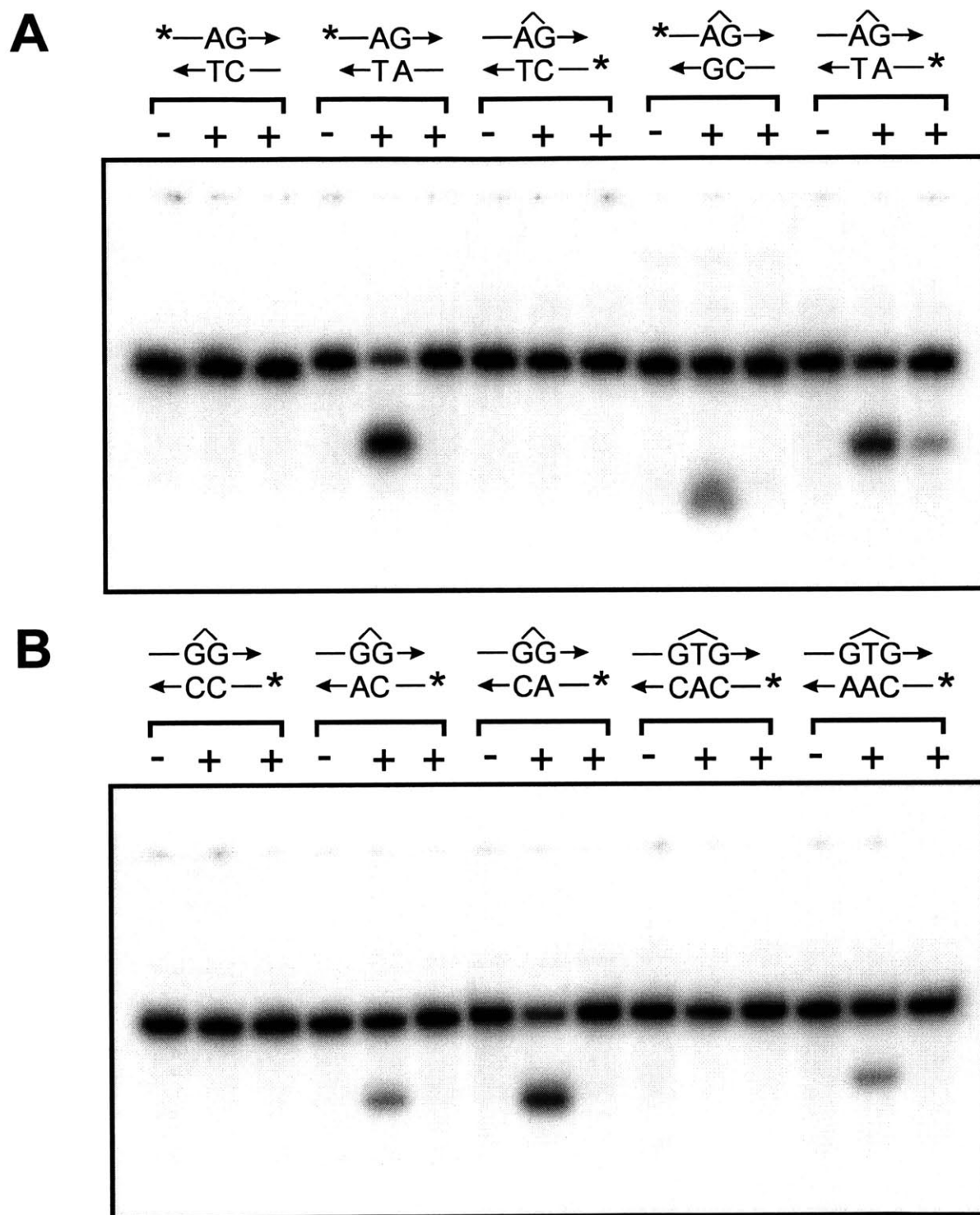
Figure 3.11: Glycosylase activity assay of p26 and p26 D138N on cisplatin compound lesions. **(A)** Unmodified DNA (Lanes 1, 2 and 3), A: G mismatch containing oligonucleotide (Lanes 4, 5, and 6), 1,2-d(ApG) oligonucleotide (Lanes 7, 8, and 9), 1,2-d(ApG/GpC) oligonucleotide (Lanes 10, 11, and 12), or 1,2-d(ApG/TpA) oligonucleotide (Lanes 13, 14, and 15) were incubated with 480 nM p26 (Lanes 2, 5, 8, 11, and 14) or with 480 nM D138N p26 (Lanes 3, 6, 9, 12, and 15). **(B)** 1,2-d(GpG) oligonucleotide (Lanes 1, 2, and 3), 1,2-d(GpG/ApC) oligonucleotide (Lanes 4, 5, and 6), 1,2-d(GpG/CpA) oligonucleotide (Lanes 7, 8, and 9), 1,3-d(GpTpG) oligonucleotide (Lanes 10, 11, and 12), or 1,3-d(GpTpG/ApApC) oligonucleotide (Lanes 13, 14, and 15) were incubated with 480 nM p26 (Lanes 2, 5, 8, 11, and 14) or with 480 nM D138N p26 (Lanes 3, 6, 9, 12, and 15). AP sites were cleaved by treatment with 0.1 N NaOH at 70°C for 20 min. The asterisk (*) denotes the ^{32}P labeled strand.
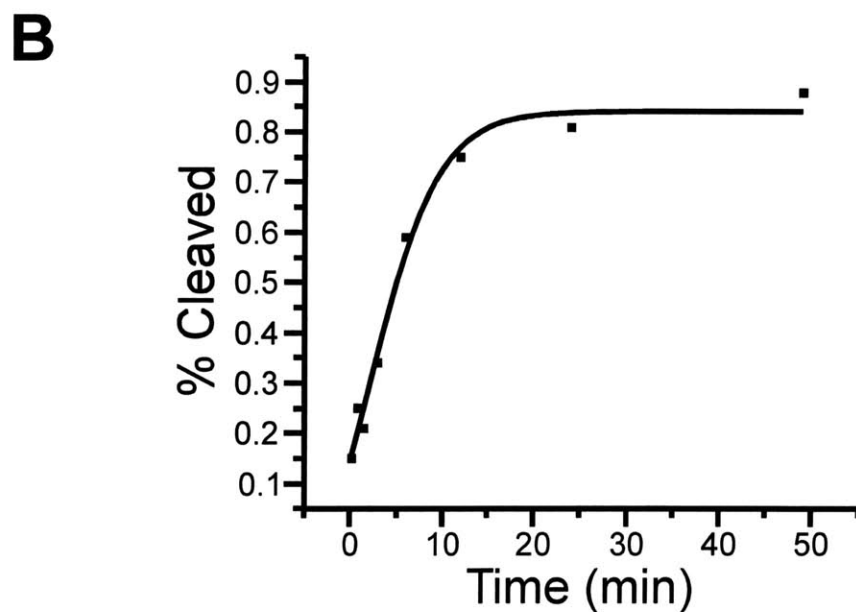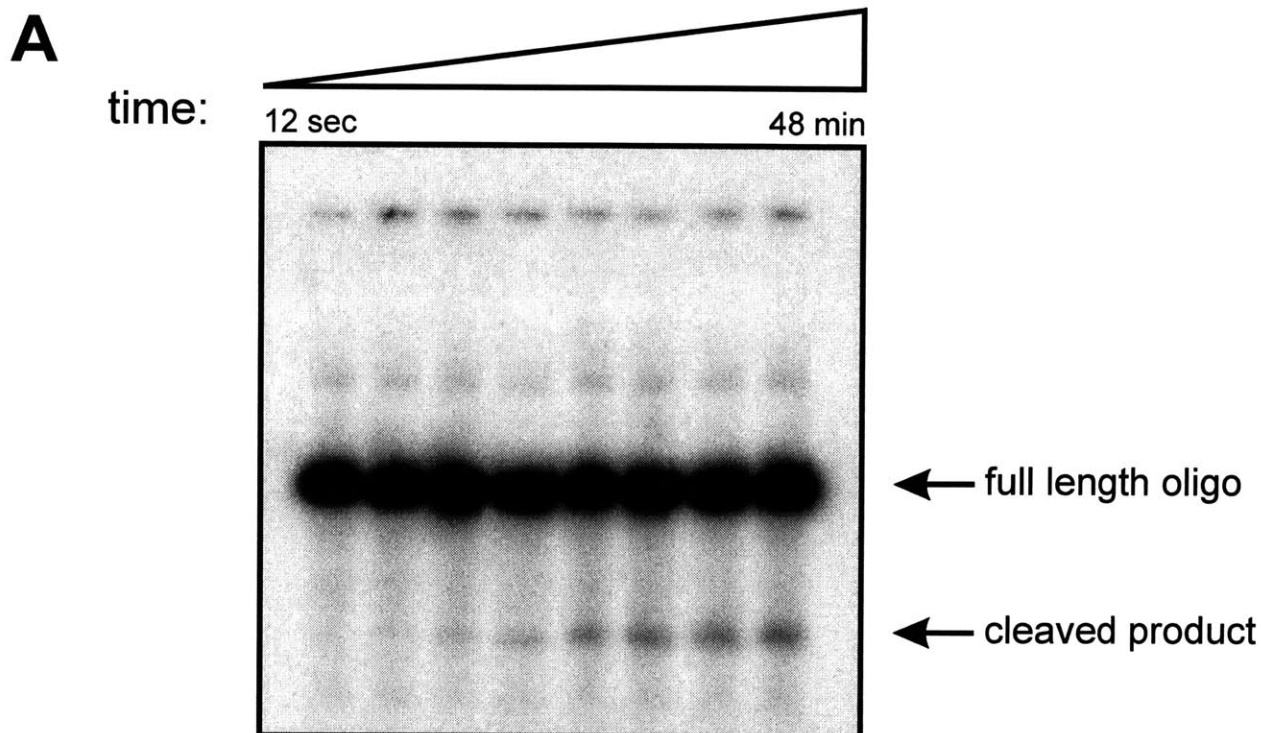
Figure 3.12: Kinetic analysis of the interaction of D138N p26 with the 1,2-d(ApG/TpA) oligonucleotide. **(A)** ^{32}P-labeled 1,2-d(ApG/TpA) oligonucleotide was incubated with 200 nM D138N p26 for 12 sec, 45 sec, 90 sec, 3 min, 6 min, 12 min, 24 min, or 48 min. Reactions were terminated by addition of 0.1 N NaOH. Base treatment effects strand scission at AP sites yielding a smaller ^{32}P-labeled DNA fragment that can be separated from the full length oligonucleotide by denaturing gel electrophoresis. **(B)** The extent of cleavage was quantitated and plotted as a function of time.
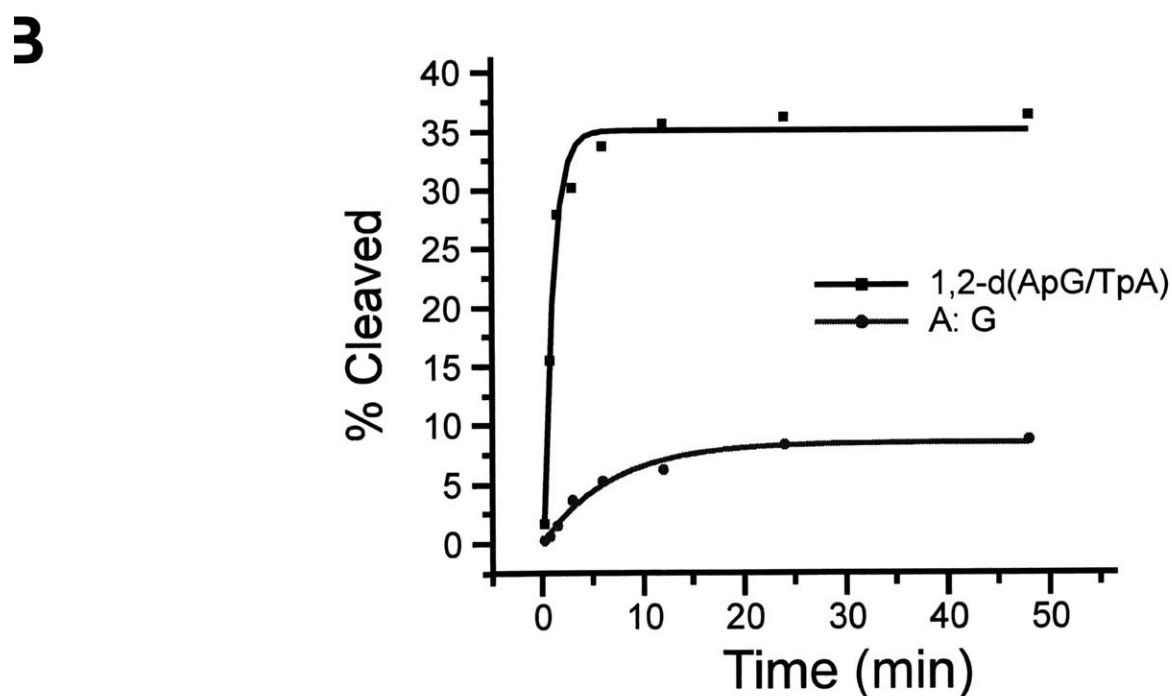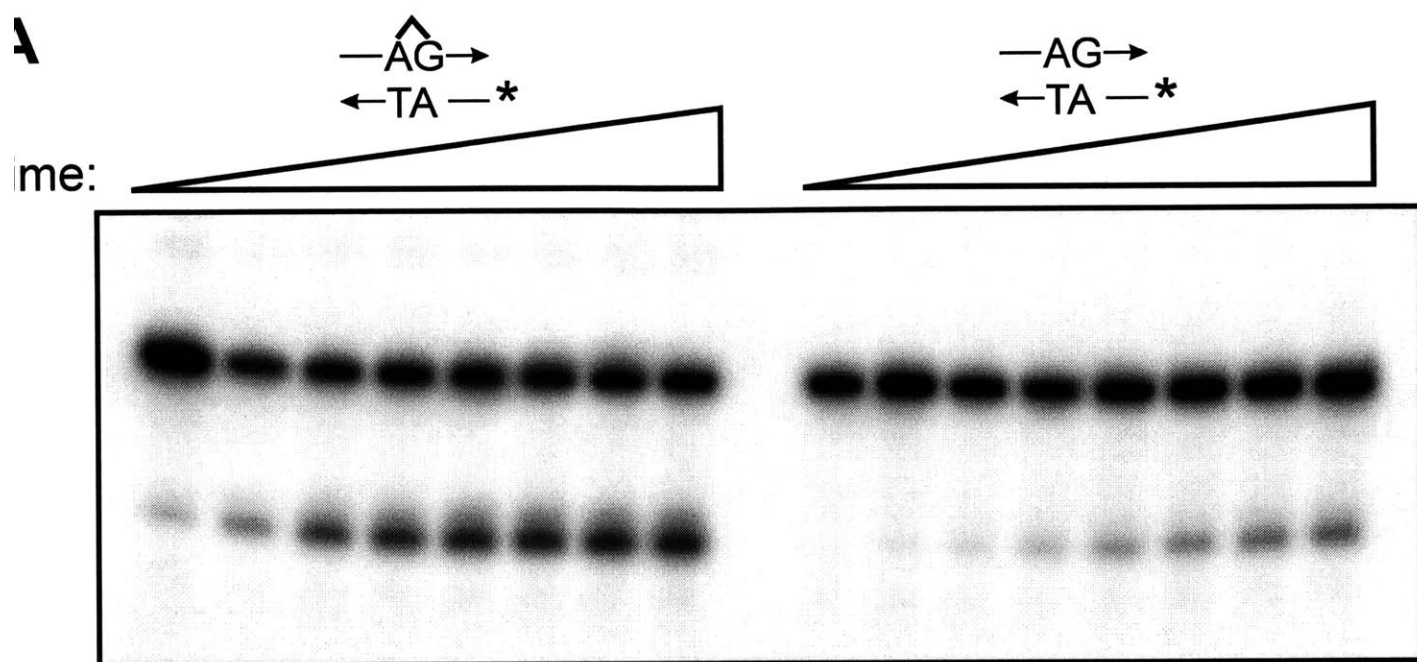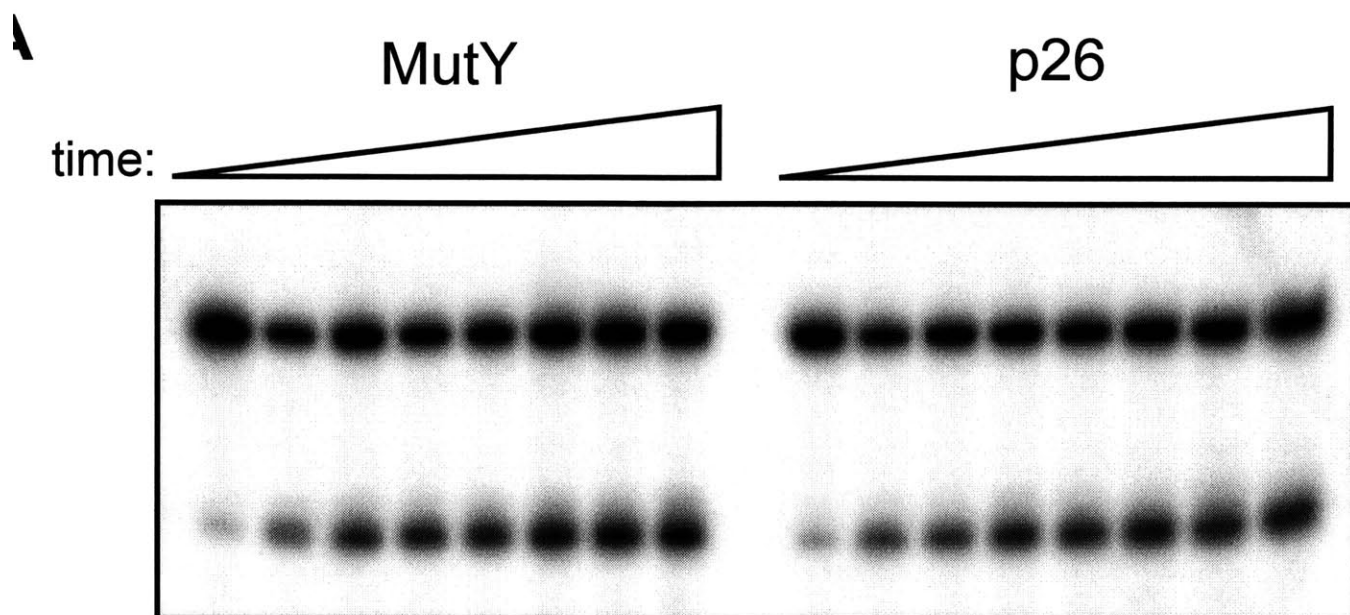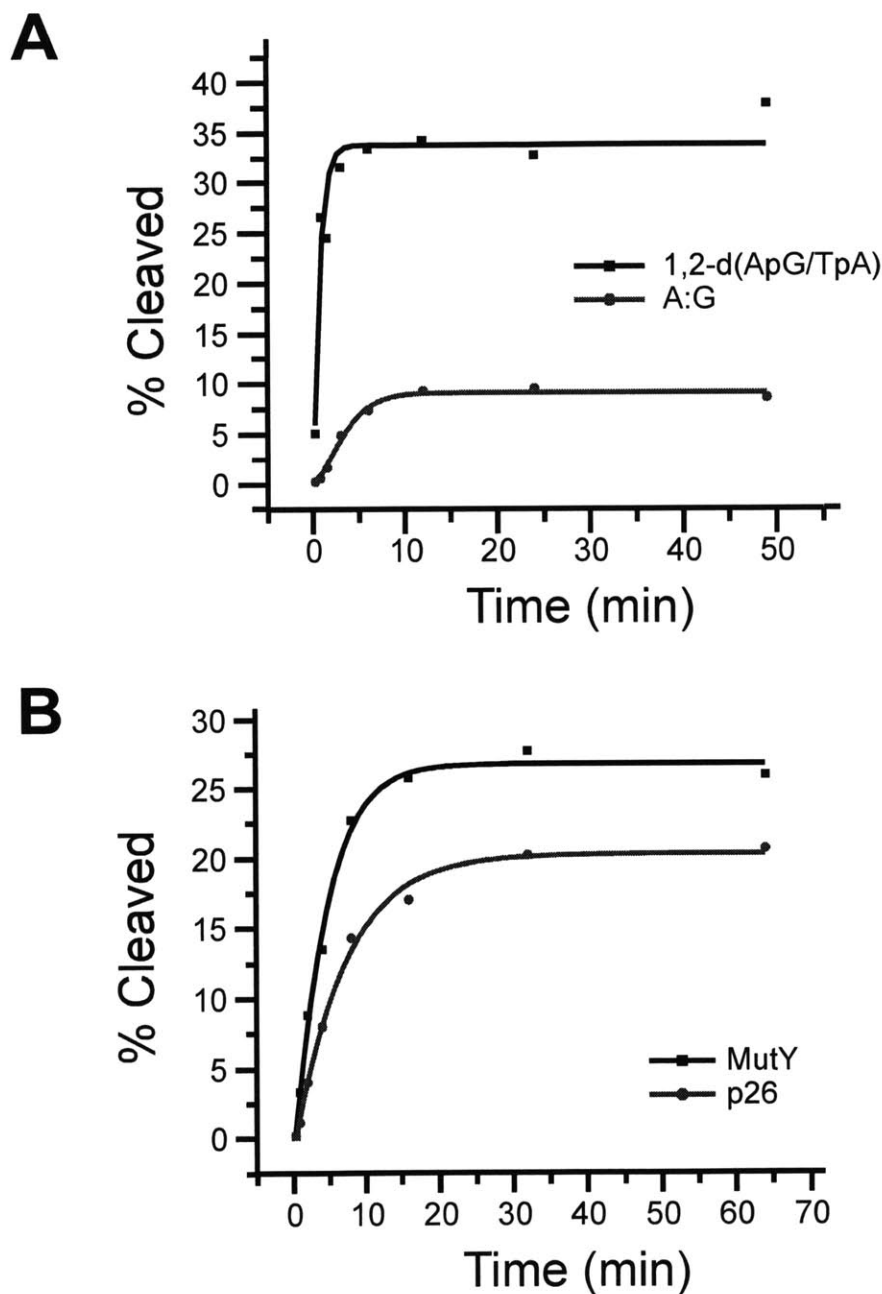
Figure 3.13: Kinetic analysis of the interaction of MutY with 1,2-d(ApG/TpA) and the A: G mismatch. **(A)** ^{32}P-labeled 1,2-d(ApG/TpA) oligonucleotide (Lanes 1 to 8) or ^{32}P-labeled A: G oligonucleotide (Lanes 10 to 17) were incubated with 200 nM MutY for 12 sec, 45 sec, 90 sec, 3 min, 6 min, 12 min, 24 min, or 48 min. Reactions were terminated by addition of 0.1 N NaOH. Base treatment effects strand scission at AP sites yielding a smaller ^{32}P-labeled DNA fragment that can be separated from the full length oligonucleotide on a denaturing polyacrylamide gel. **(B)** The extent of cleavage was quantitated and plotted as a function of time.
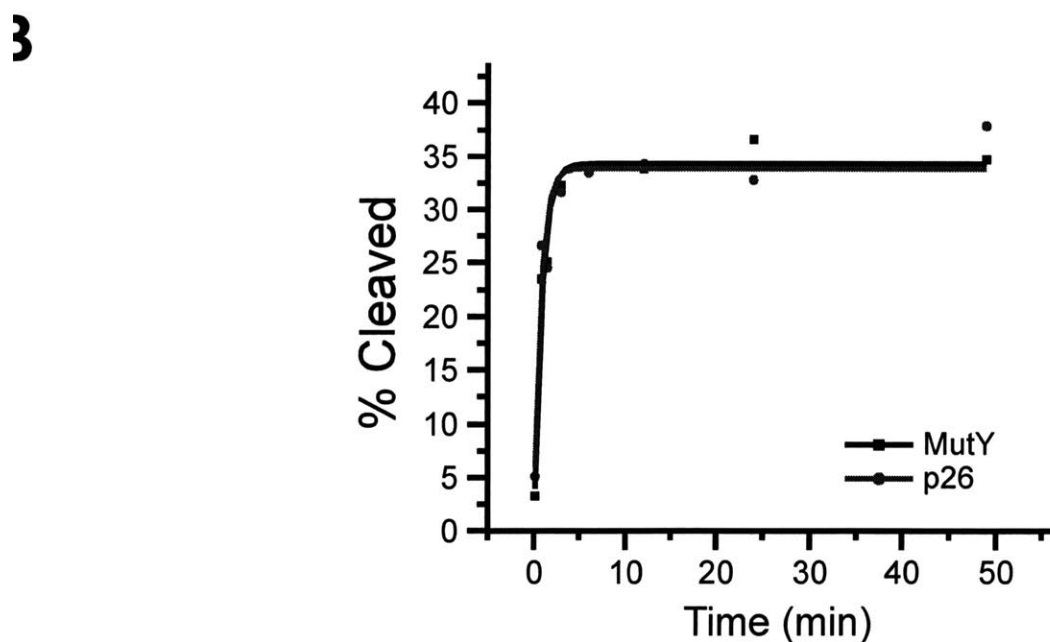
Figure 3.14: Kinetic analysis of MutY and p26 with 1,2-d(ApG/TpA). **(A)** ^{32}P-labeled 1,2-d(ApG/TpA) oligonucleotide was incubated with 200 nM MutY (lanes 1 to 8) or 200 nM p26 (Lanes 10 to 17) for 12 sec, 45 sec, 90 sec, 3 min, 6 min, 12 min, 24 min, or 48 min. Reactions were terminated by addition of 0.1 N NaOH. Base treatment effects strand scission at AP sites yielding a smaller ^{32}P-labeled DNA fragment that can be separated from the full length oligonucleotide by denaturing gel electrophoresis. **(B)** The extent of cleavage was quantitated and plotted as a function of time.

Figure 3.15: **(A)** Kinetic analysis of the interaction of p26 with 1,2-d(ApG/TpA) and the A: G mismatch. ^{32}P-labeled 1,2-d(ApG/TpA) oligonucleotide or ^{32}P-labeled A: G oligonucleotide were incubated with 200 nM p26 for 12 sec, 45 sec, 90 sec, 3 min, 6 min, 12 min, 24 min, or 48 min. The extent of cleavage 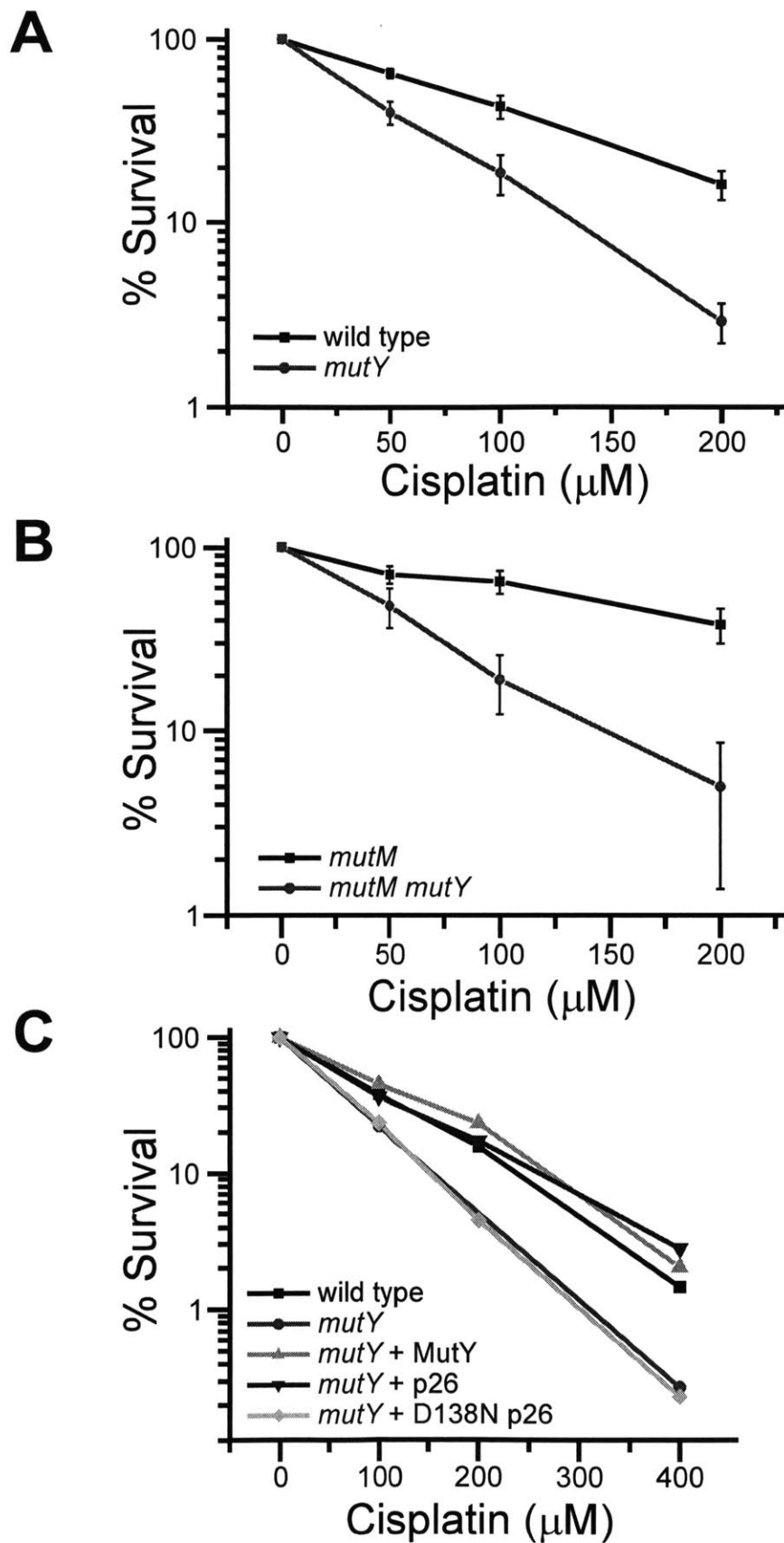was quantitated and plotted as a function of time. **(B)** Kinetic analysis of MutY and p26 with the A: G mismatch. ^{32}P-labeled A: G oligonucleotide was incubated with 200 nM MutY or 200 nM p26 for 12 sec, 45 sec, 90 sec, 3 min, 6 min, 12 min, 24 min, or 48 min. The extent of cleavage was quantitated and plotted as a function of time.

Figure 3.16: **(A)** Survival of wild type and *mutY* cells after treatment with cisplatin. **(B)** Survival of *mutM* and *mutM mutY* cells after treatment with cisplatin. **(C)** Survival of wild type, *mutY*, and mutY cells expressing MutY, p26, or D138N p26 after treatment with cisplatin. The data in these graphs are the average of at least four experiments and the error bars represent the standard error of the mean.
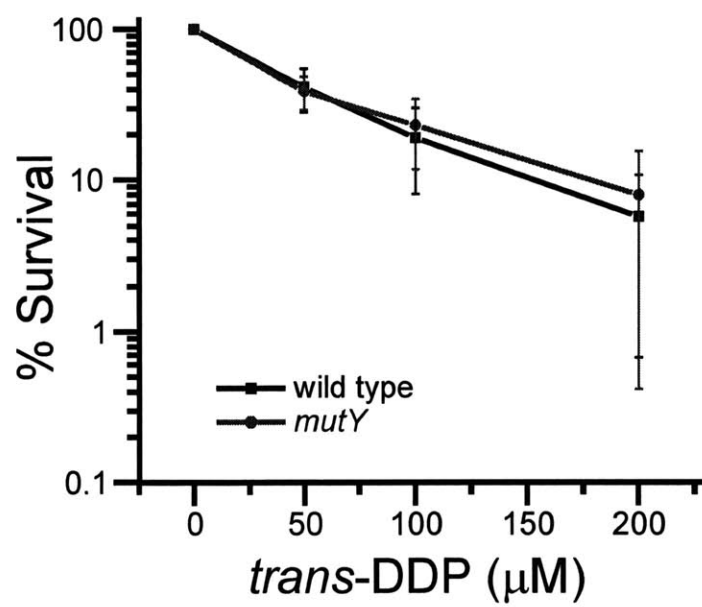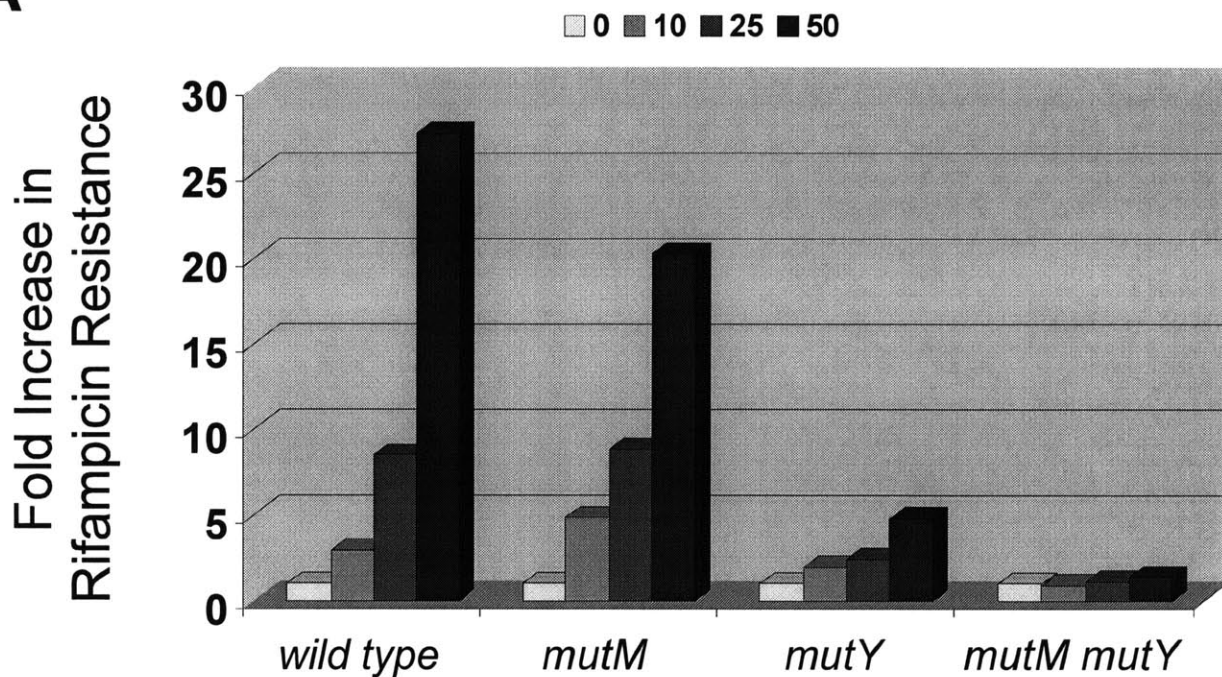
Figure 3.17: Survival of wild type and *mutY* cells after treatment with *trans*-DDP. The data in this graph are the average of four experiments and the error bars represent the standard error of the mean.
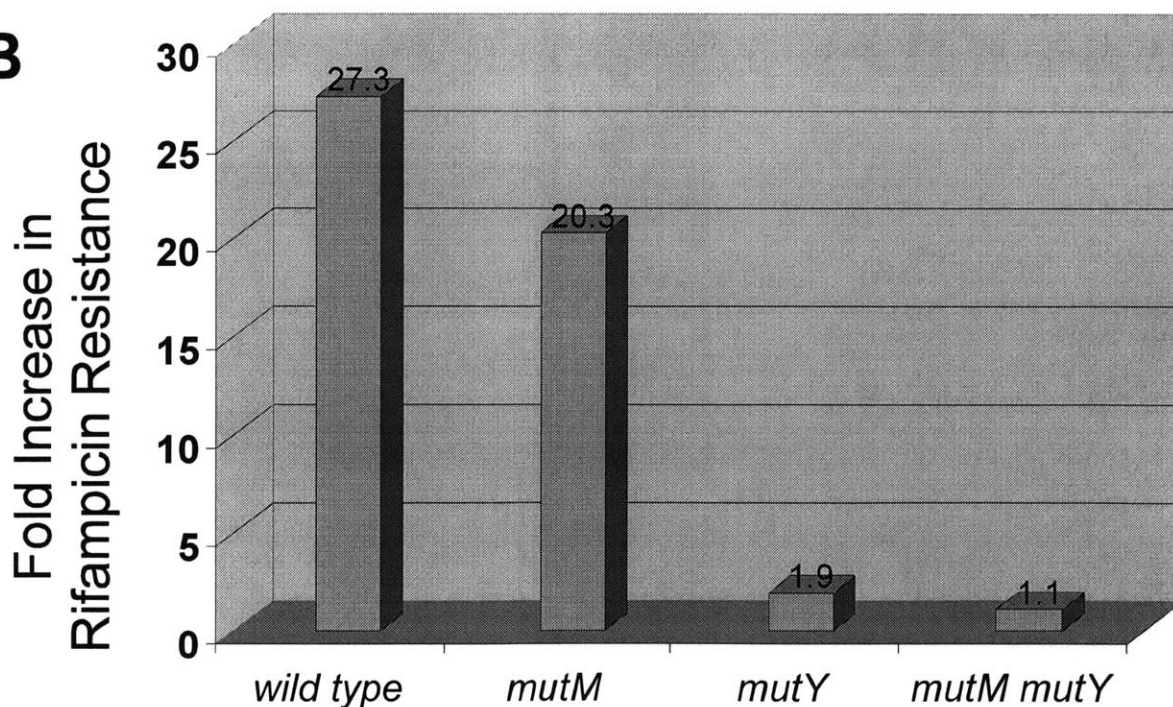
Figure 3.18: Mutagenic potency of cisplatin. **(A)** Fold increase in number of rifampicin resistant cells after treatment of wild type, *mutM*, *mutY*, or *mutM mutY* cells with 0, 10, 25 or 50 μM cisplatin. **(B)** Fold increase in number of rifampicin resistant cells after treatment of wild type, *mutM*, *mutY,* or *mutM mutY* cells with cisplatin doses that result in 70% survival.
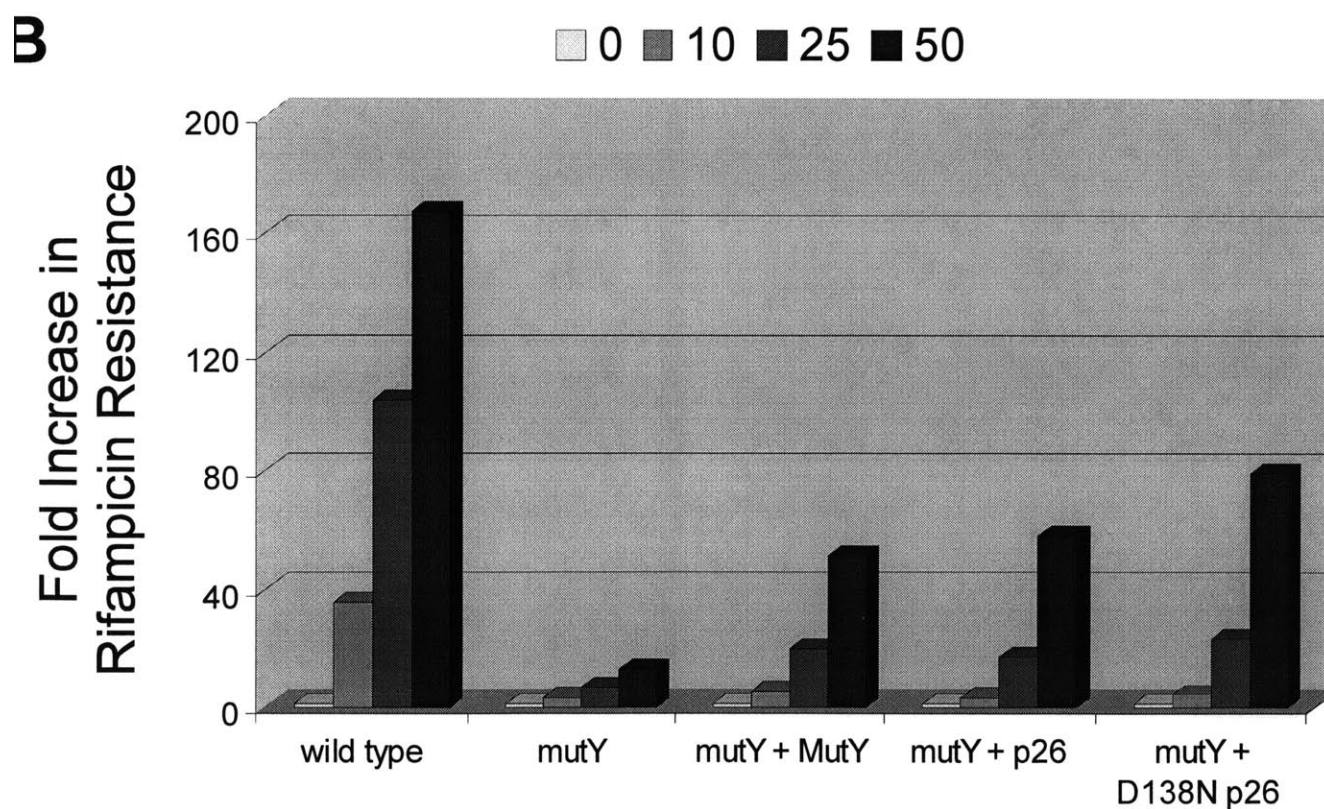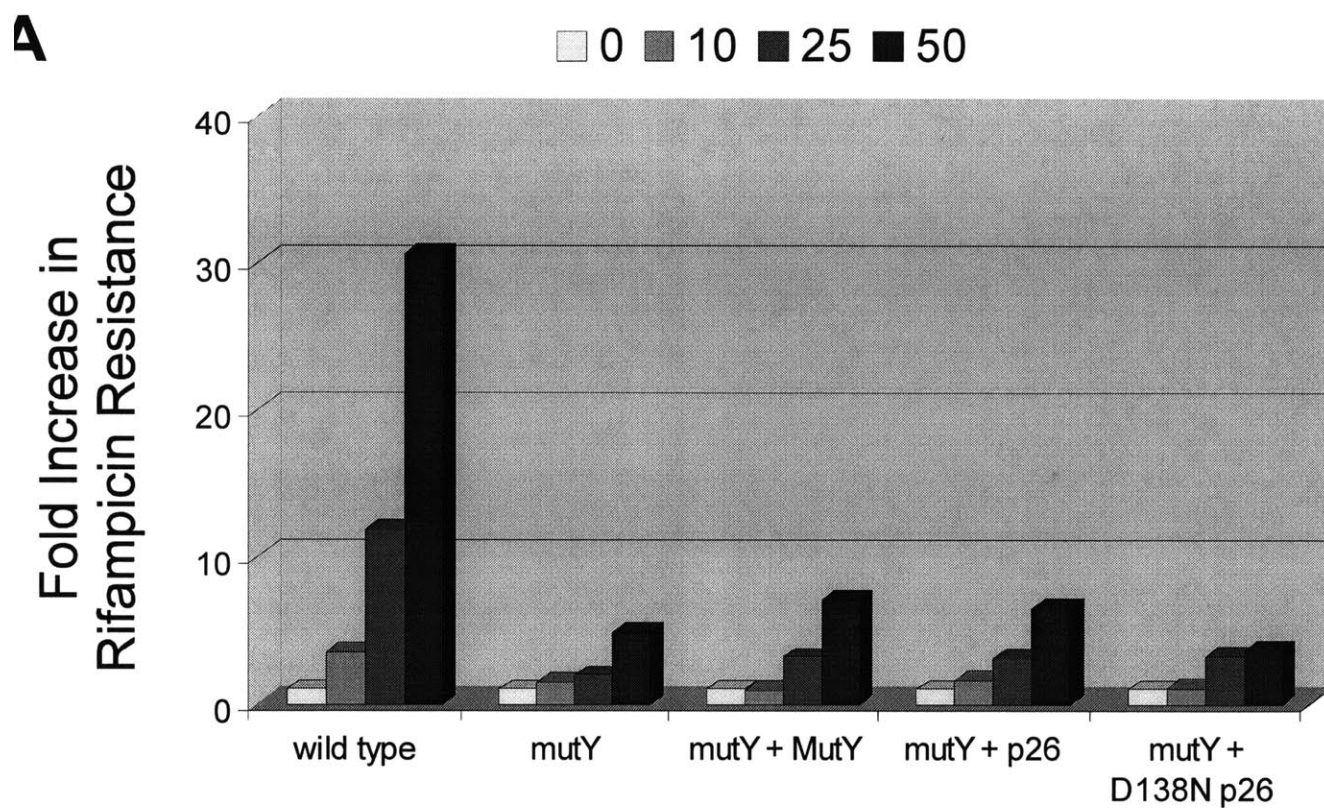
Figure 3.19: Mutagenic potency of cisplatin in *mutY* cells expressing MutY, p26, or D138N p26 under **(A)** non-inducing conditions, or **(B)** induction with 100 μM IPTG. Wild type, *mutY,* and *mutY* cells expressing MutY, p26, or D138N p26 were treated with cisplatin for **(A)** 1 hr at 37°C or **(B)** 1 hr at 37°C and 30 min at room temperature and the fold increase in rifampicin resistant cells was determined.
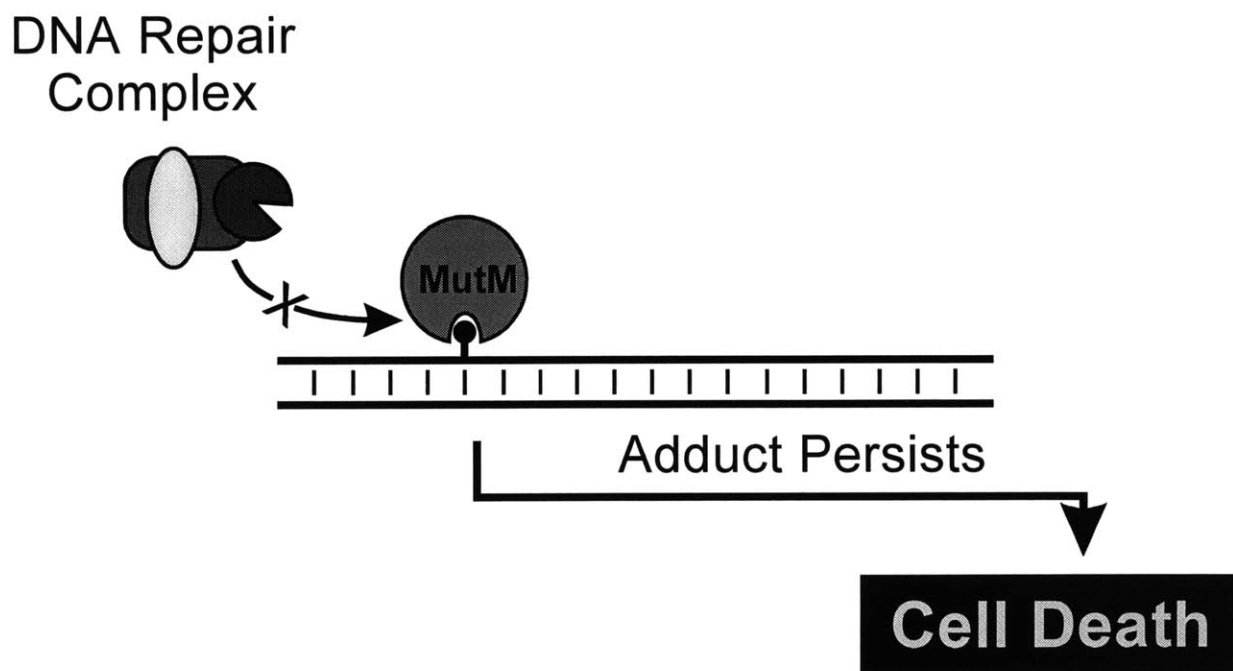
Figure 3.20: MutM shields cisplatin adducts from repair. MutM recognizes cisplatin adducts and can inhibit their repair by physically blocking the access to other repair proteins. The cisplatin adducts persist in DNA, thereby potentiating their toxicity.
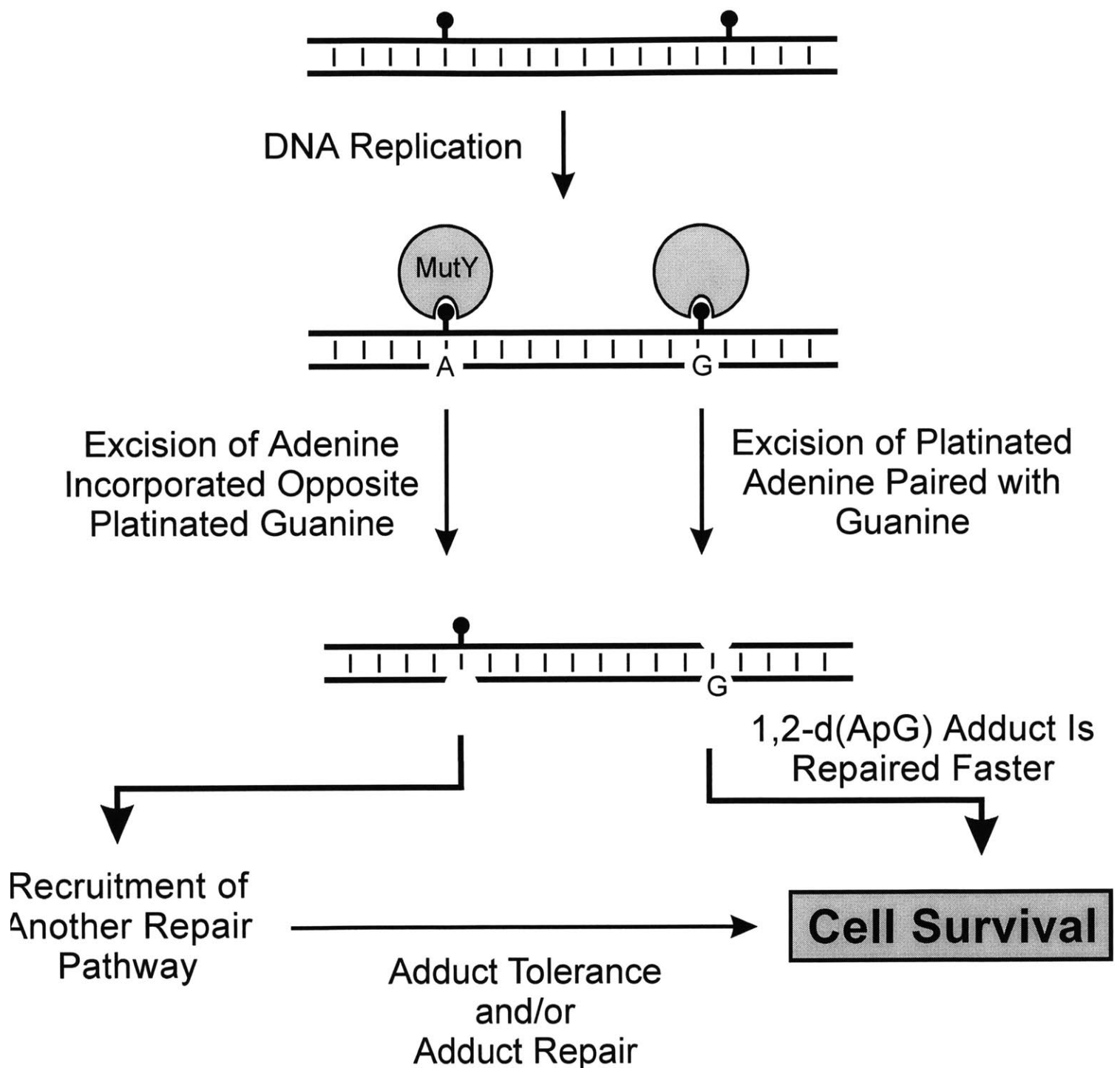
Figure 3.21: MutY protects cells from cisplatin toxicity. MutY can remove the 1,2-d(ApG) adduct of cisplatin and thereby reduce cisplatin toxicity. Moreover, MutY can generate a strand break opposite the adduct, which serves as a signal for another repair pathway. The adducts are repaired faster or they are better tolerated by the cell, and their cytotoxic effects are diminished.
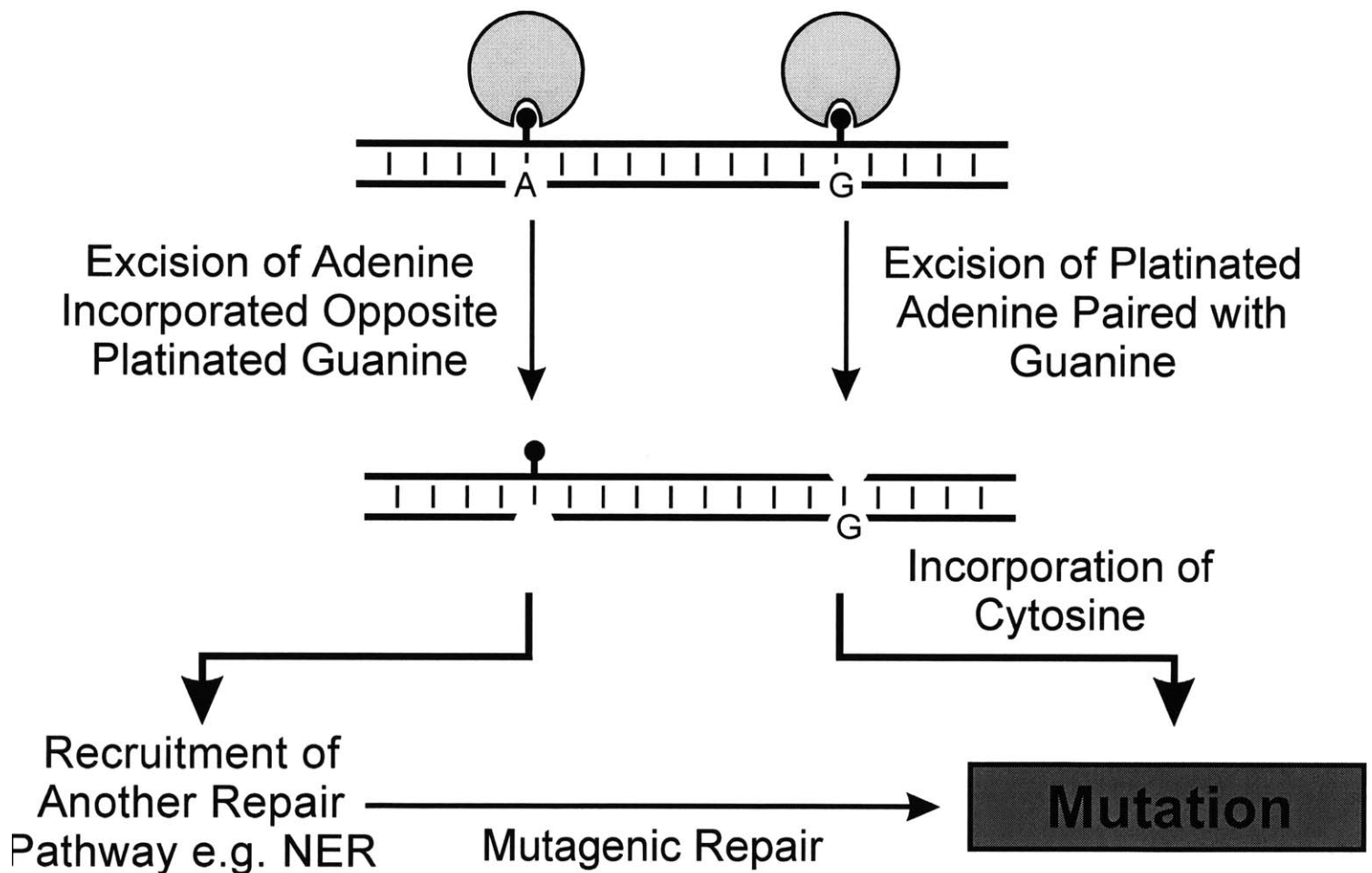
A

Figure 3.22: MutY increases the mutagenic potency of cisplatin. **(A)** After DNA replication an adenine or a guanine is inserted opposite cisplatin adducts, making them substrates for MutY. The action of MutY on cisplatin adducts causes a more efficient transfer to nucleotide excision repair proteins. The activity of nucleotide excision repair proteins can be mutagenic. In the absence of MutY, the adducts get transferred to nucleotide excision repair proteins less efficiently, or they die before a mutation has arisen. Moreover, removal of the 1,2-d(ApG) adduct when mispaired with guanine and resynthesis across the gap leads to an A to C mutation.
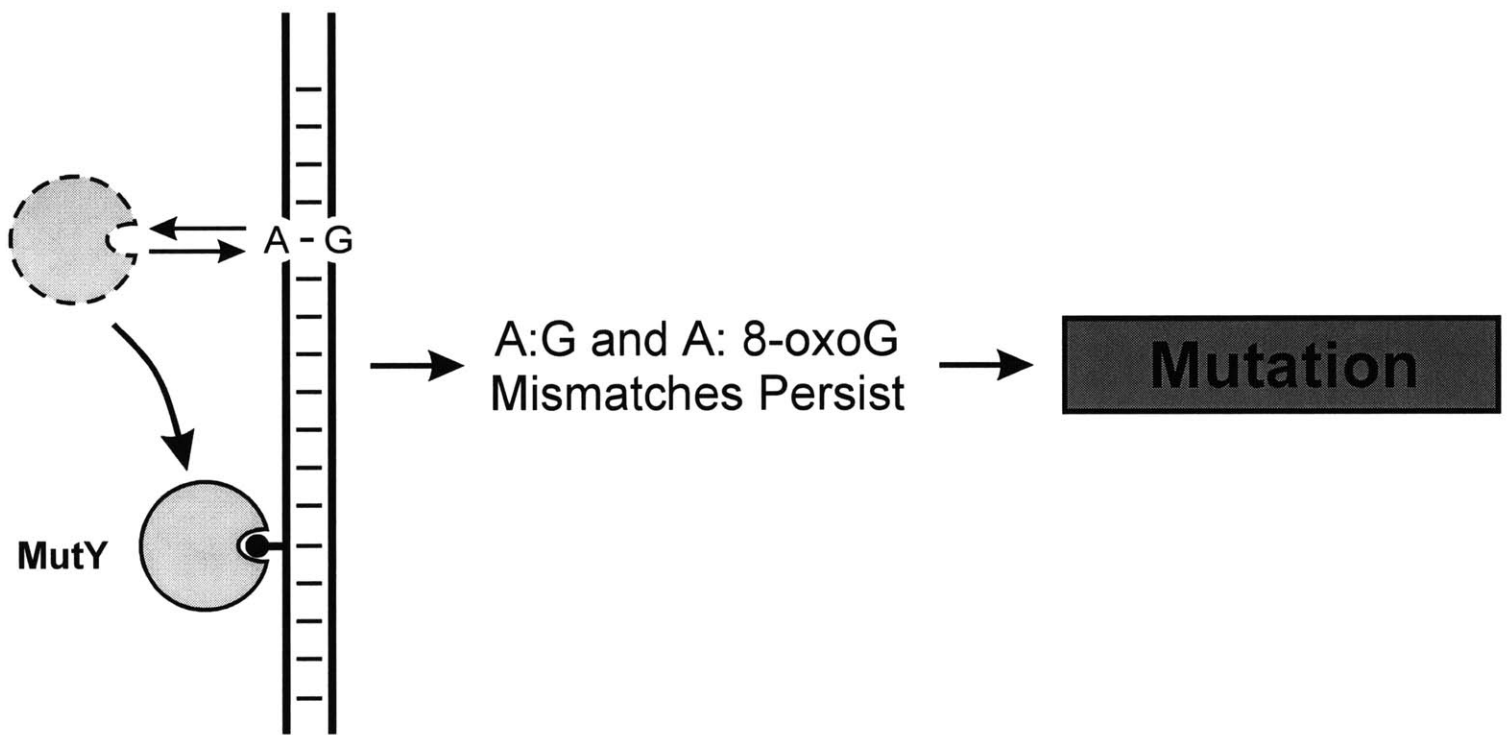
B

Figure 3.22: MutY increases the mutagenic potency of cisplatin. **(B)** The repair factor hijacking model. MutY can interact and repair A: G or A: 8-oxoG substrates or it can interact with cisplatin adducts. In a cisplatin treated cell the concentration of MutY available to repair A: G or A: 8-oxoG is reduced since some of the MutY protein is in a complex with the cisplatin adducts. The repair of A: G and A: 8-oxoG mismatches is less efficient and the lesions persist in DNA, thereby promoting mutagenesis.

Figure 3.23: D138N p26 can increase the mutagenic potency of cisplatin adducts. When a guanine is misincorporated opposite adenine during DNA replication, MutY and MutS compete for binding to the A: G mismatch. **(A)** When MutS binds to the mismatch, the guanine gets removed restoring the original sequence, whereas when MutY binds to the mismatch, the adenine gets removed causing a mutation.
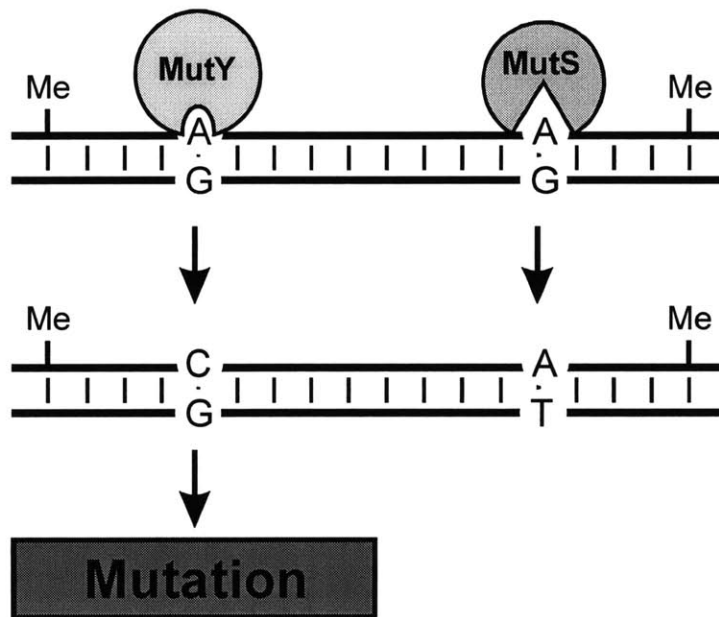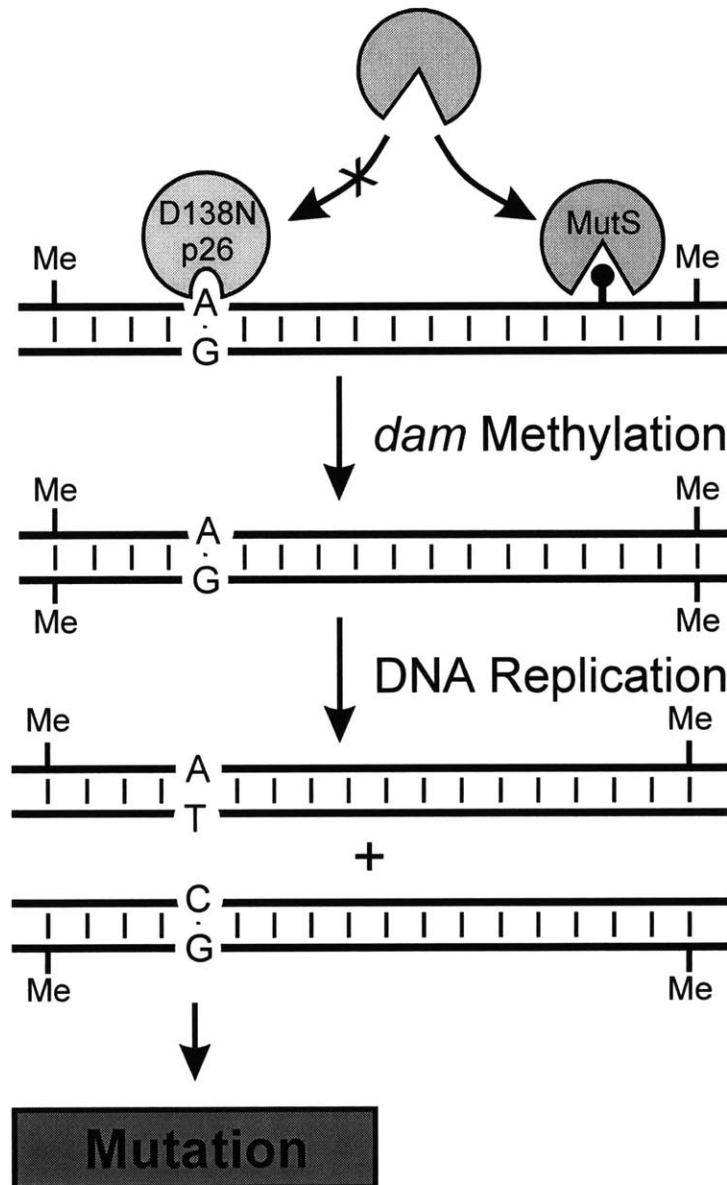
Figure 3.23: D138N p26 can increase the mutagenic potency of cisplatin adducts. When a guanine is misincorporated opposite adenine during DNA replication, MutY and MutS compete for binding to the A: G mismatch. **(B)** In *mutY* cells overexpressing D138N p26, the D138N p26 protein binds to the A: G mismatch and physically blocks the access to the mismatch. Moreover, binding of MutS to cisplatin adducts decreases the amount of MutS protein available to displace D138N p26 from the mismatch. After *dam* methylation has taken place, the strand discrimination signal is lost, and MutS is no longer able to repair the A: G mismatch. In the next round of replication the mutation is fixed.

Appendix A

The Role of Mismatch Repair in Cisplatin Toxicity

Role of mismatch repair in cisplatin resistance

A study with *E. coli* cells demonstrated that *dam* cells, which lack the methylase activity responsible for the methylation of adenine at GATC sites, are more sensitive to cisplatin than wild type cells, and introduction of a second mutation at the *mutS* or *mutL* genes abrogates the sensitivity of this strain, suggesting that mismatch repair is involved in the cellular responses to cisplatin (Fram et al., 1985). Interestingly, the clinically ineffective *trans*-DDP is equally toxic to wild type and *dam* strains. These results led us to hypothesize that mismatch repair proteins would have selective affinity for cisplatin adducts and that mammalian cells defective in mismatch repair would be more resistant to cisplatin than the corresponding wild type cells.

Because sets of isogenic cell lines proficient and deficient in mismatch repair were unavailable at the time, we determined the responses of a panel of colorectal cancer cell lines after treatment with cisplatin. All cell lines were obtained from the American tissue type collection. The HT29 cell line was derived from a human colon adenocarcinoma, and the SW620 was derived from the lymph node metastasis of a colon adenocarcinoma, and both are wild type with respect to mismatch repair. HT29 cells express wild type p53 whereas the SW620 cells have a mutation in the p53 gene. The HT29 cells express high levels of O^6 methylguanine methyltransferase (MGMT), and are resistant to *N*-methyl-*N*-nitrosourea (MNU), whereas SW620 cells do not express detectable amounts of MGMT, and are sensitive to MNU. The LoVo cell line was derived from a colon adenocarcinoma and is *hMSH2* deficient. Moreover, since the hMSH6 protein is not stable in the absence of the hMSH2 protein, LoVo cells do not express any detectable hMSH6. Nuclear extracts from these cells are unable to repair DNA containing a single mismatch, as well as heteroduplexes

containing one to five extra bases, and this defect is restored by addition of purified hMutSα. The HCT116 cell line was derived from a human colon carcinoma, expresses wild type p53 and is *hMLH1* deficient. HCT116 cells are resistant to MNNG, MNU, and 6-thioguanine. Nuclear extracts from these cells are unable to repair small loops but are efficient in the repair of 5, 8 and 16 base loops. The DLD1 cell line was derived from a human colon adenocarcinoma, is resistant to MNU, and is *hMSH6* deficient. DLD1 cells also contain a mutation in the polymerase δ gene. Nuclear extracts from DLD1 cells are unable to bind to a G: T mismatch even though they expresses wild type hMSH2 protein.

The survival of SW620, HT29, LoVo, HCT116, and DLD1 cells after treatment with cisplatin was determined in a clonogenic assay. Various dilutions of cells were plated onto 6-well plates, and were treated with various concentrations of cisplatin. Cells were allowed to grow for 2 weeks, colonies were fixed in ethanol: acetic acid 3:1, stained with crystal violet, and counted. The HT29 cells were slightly more resistant to cisplatin than the SW620 cells. Among these cell lines, the LoVo cells were the most resistant to cisplatin. For example, the HT29 and SW620 cells were 10 fold more sensitive to cisplatin than the LoVo cells. Interestingly, the LoVo cells were also more resistant to *trans*-DDP than HT29 cells. Finally, the DLD1 and the HCT116 cells were more resistant to cisplatin than the SW620 cells.

Interestingly, when the survival of cells after treatment with cisplatin was determined in a clonogenic assay the mismatch repair proficient HT29 cells were more sensitive to cisplatin than the mismatch repair deficient LoVo cells, whereas when survival was measured in growth inhibition

assays the results varied depending on the time of treatment. If survival was determined within a couple of days after treatment, the HT29 cells appeared to be more resistant to cisplatin than the LoVo cells. In contrast, at later time points the HT29 cells appeared to be more sensitive than the LoVo cells which was consistent with the results from the clonogenic assays. These observations led us to hypothesize that initial treatment with cisplatin was more toxic to LoVo cells than to HT29 cells and that HT29 cells arrested in the cell cycle and eventually died following treatment, whereas the LoVo cells continued to cycle. To test this hypothesis, we measured the distribution of cells in each phase of the cell cycle after treatment with cisplatin by flow cytometry. Consistent with our hypothesis, the LoVo cells failed to arrest in the G2 phase of the cell cycle, whereas the HT29 cells arrested at G2. Interestingly, the HCT116 and DLD1 cells were also able to arrest at G2 after cisplatin treatment, indicating that the failure to arrest at G2 after cisplatin treatment is not a general feature of mismatch repair deficient cell lines. In contrast Brown et al. (Brown et al., 1997) demonstrated that an ovarian carcinoma cell line that has acquired resistance to cisplatin *in vitro,* is hMLH1 deficient, and has lost the ability to arrest at the G2 phase of the cell cycle after cisplatin treatment. This cell line, however, is also p53 deficient indicating that the mismatch repair deficiency might not be responsible for the failure to arrest at G2, and underscoring the importance of using isogenic cell lines in these experiments.

Test whether hMSH2 expression is controlled by p53

The p53 protein is a transcriptional activator; it can bind to specific DNA sequences located upstream of certain genes, and can activated the transcription of these genes. Interestingly, examination of the promoter region of the *hMSH2* gene revealed a sequence with high homology to

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the p53 consensus binding sequence. Moreover, *in vitro* experiments with purified p53 showed that p53 can bind to this sequence, indicating that *hMSH2* might be a p53 regulated gene. To determine whether cisplatin treatment, which induces p53 expression, can also induce hMSH2 expression we treated mismatch repair proficient HT29 cells with cisplatin and we measured the levels of p53 and hMSH2 proteins by western blot analysis. Cisplatin treatment caused an increase in p53 levels. In contrast, no increase in the hMSH2 expression was observed under these conditions. Further experiments need to be carried out in order to verify this observation.

Interaction of MutS with cisplatin adducts

Purified MutS protein was obtained from Genecheck. The oligonucleotides 5'-GATCCGTCGACCTGCA-3' and 5'-TGCAGGTTGACGGATC-3', where the bases involved in the G: T mismatch are indicated in bold, were obtained from Dave Wang, the oligonucleotide 5'-TCTCCTTCTGGTCTCTTCTC-3', where the platinated bases are indicated in bold, was obtained from Jill Mello and the oligonucleotide 5' GAGAAGAGACCAGAAGGAGA-3' was obtained from Elizabeth Trimmer. The reaction buffer contained 20 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, pH 8.0, 0.1 mM DTT, and 6.3 ng/μl MutS. Under these conditions MutS readily recognized the G: T mismatch but was unable to recognize the 1,2-d(GpG) cisplatin adduct. Further experiments carried out by Zoran Zdravevski in our lab indicate that MutS can recognize cisplatin adducts under different reaction conditions (Z. Zdravevski, manuscript in preparation).

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Appendix B

MutM Can Remove 5-hydroxyuracil and 5-hydroxycytosine from DNA

Repair of 5OHC and 5OHU by MutM

Oligonucleotides containing 5-hydroxycytosine ($5^{OH}C$) or 5-hydroxyuridine ($5^{OH}U$) were synthesized and characterized by David Wang (Wang and Essigmann, 1997). These oligonucleotides were ^{32}P-labeled, annealed to complementary oligonucleotides containing guanine opposite the lesion and used in glycosylase assays with purified endonuclease III or MutM proteins. Endonuclease III was able to remove both lesions as previously reported (Wang and Essigmann, 1997). Interestingly, MutM was also able to excise both lesions. Time course experiments demonstrated an increase in cleaved product over time. Preliminary experiments to determine K_{cat} and K_M values for excision of $5^{OH}C$ and $5^{OH}U$ were initiated.

Karakaya et al. (Karakaya et al., 1997)revealed that $5^{OH}C$ and $5^{OH}U$ are not released by MutM as determined by gas chromatography mass spectrometry (GC-MS). Subsequent studies by D'Ham et al. (D'Ham et al., 1999) demonstrate that MutM can cleave oligonucleotides containing $5^{OH}C$, but it does not release free $5^{OH}C$ ad determined by GC-MS, probably because $5^{OH}C$ is chemically modified during the excision reaction.

References

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Appendix C

Ithaka by Kavafy

ITHAKA by Cavafy

As you set out for Ithaka

hope the voyage is a long one,

full of adventure, full of discovery,

Laistrygonians and Cyclops,

angry Poseidon— don't be afraid of them:

you'll never find things like that on your way

as long as you keep your thoughts raised high,

as long as a rare excitement

stirs your spirit and your body.

Laistrygonians and Cyclops,

wild Poseidon — you won't encounter them

unless you bring them along inside your soul,

unless your soul sets them up in front of you.

Hope the voyage is along one,

May there be many a summer morning when,

with what pleasure, what joy,

you come into harbors seen for the first time;

may you stop at Phoenician trading stations

to buy fine things,

mother of pearl and coral, amber and ebony,

sensual perfume of every kind —

as many sensual perfumes as you can;

and may you visit many Egyptian cities

to gather stores of knowledge from their scholars.

Keep Ithaka always in your mind.

Arriving there is what you are destined for.

But do not hurry the journey at all.

Better if it lasts for years,

so you are old by the time you reach the island,

wealthy with all you have gained on the way,

not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.

Without her you would not have set out.

She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.

Wise as you will have become, so full of experience,

you will have understood by then what these Ithakas mean.

Ιθάκη (Καβάφης 1911)

Σα βγείς στον πηγαιμό για την Ιθάκη

να εύχεσαι νάναι μακρύς ο δρόμος

γεμάτος περιπέτειες γεμάτος γνώσεις

Τους Λαιστρυγόνας και τους Κύκλωπας

τον θυμωμένο Ποσειδώνα μη φοβάσαι

τέτοια στον δρόμο σου ποτέ σου δεν θα βρεις

αν μέν η σκέψις σου υψηλή αν εκλεκτή

συγκίνησις το πνεύμα και το σώμα σου αγγίζει

Τους Λαιστρυγόνας και τους Κύκλωπας

τον άγριο Ποσειδώνα δεν θα συναντήσεις

αν δεν τους κουβανείς μες στην ψυχή σου

αν η ψυχή σου δεν τους στήνει εμπρός σου

Ν α εύχεσαι νάναι μακρύς ο δρόμος

Πολλά τα καλοκαιρινά πρωιά να είναι

που με τι ευχαρίστησι με τι χαρά

θα μπαίνεις σε λιμένας πρωτοειδωμένους

να στ αματήσεις σ εμπόρεια φοινικικά

και τες καλές πραγμάτειες ν αποκτήσεις

σεντέφια και κοράλλια κεχριμπάρια κ έβενους

και ηδονικά μυρωδικά κάθε λογής

όσο μπορείς πιο άφθονα ηδονικά μυρωδικά

σε πόλεις Αιγυπτιακές πολλές να πας

να μάθεις και να μάθεις από τους σπουδασμένους

Πάντα στον νου σου νάχεις την Ιθάκη

Το φθάσιμον εκεί είναι ο προορισμός σου

Αλλά μην βιάζεις το ταξείδι διόλου

Καλλίτερα χρόνια πολλά να διαρκέσει

και γέρος πια ν αράξεις στο νησί

πλούσιος με όσα κέρδισες στον δρόμο

μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη

Η Ιθάκη σ έδωσε τ ωραίο ταξείδι

Χωρίς αυτήν δεν θάβγαινες στον δρόμο

Άλλα δεν έχει να σε δώσει πια

Κι αν πτωχική την βρεις η Ιθάκη δεν σε γέλασε

Έτσι σοφός που έγινες με τόση πείρα

ήδη θα το κατάλαβες η Ιθάκες τι σημαίνουν

Biography

The author was born on August 12, 1972 to Iason and Elsa Kartalou in Athens, Greece. She grew up in Nea Smirni, a suburb of Athens, with two siblings, Athina and Aris. While growing up she studied french for 6 years and she received a "certificat de langue francaise". She also studied English and she received a proficiency degree from the University of Michigan (U.S.A.) and a proficiency degree from Cambridge University (England). Following her high school graduation she moved to Heraclion, Crete where she attended the Chemistry Department of the University of Crete. She graduated with honors and received her B.S. in Chemistry in 1994. While in the University of Crete she conducted research under the supervision of Vassilis Zannis and Dimitris Kardassis. In September 1994, she moved to the U.S. where she pursued a Ph.D. degree in Toxicology. She joined the laboratory of professor John M. Essigmann where she studied the role of base excision repair proteins in the cellular responses to the anticancer drug cisplatin and mentored two undergraduate students.