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

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Received February 23, 2000

ABSTRACT: 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. 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. Variations in tumor response to cisplatin may result from altered expression of cellular proteins that recognize cisplatin adducts. The present study focuses on the interaction between the cisplatin intrastrand cross-links and human AAG. Using site-specifically modified oligonucleotides containing each of the cisplatin intrastrand cross-links, 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 because of the persistence of AAG substrates in DNA.

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-glycosidic bond between the modified base and the sugar−phosphate backbone (*1*). 3-Methyladenine DNA glycosylases identified in eukaryotic cells include the mouse (*Aag*) and the human (AAG,[1] also known as MPG or ANPG) enzymes, which can remove 3-methyladenine (3-mA), 3-methylguanine, and 7-methylguanine from DNA (*2*, *3*). Additionally, mammalian 3-methyladenine DNA glycosylases remove hypoxanthine, possibly 7,8-dihydro-8-oxoguanine, and bases with etheno substitutions (*4*). In particular, AAG is very efficient in releasing 1,N^6-ethenoadenine (ϵA) from DNA (*5*−*10*), 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 (*11*). 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, both of which are used in cancer chemotherapy (*12*−*14*). Furthermore, the human and rat 3-methyladenine DNA glycosylases excise adducts formed by nitrogen mustards (*15*) and *Saccharomyces cerevisiae* cells deficient in 3-methyladenine DNA glycosylase are more sensitive to nitrogen mustards than the corresponding wild-type cells (*16*). Accordingly, significant effort has been put into elucidating the role of AAG as a protective agent in cells treated with a cancer chemotherapeutic agent.

cis-Diamminedichloroplatinum(II), or cisplatin (Figure 1A), is an excellent example of a successful cancer chemotherapeutic agent (*17*−*19*). It is most effective in the treatment of testicular tumors, where cisplatin-based combination chemotherapy can afford cure rates of over 90% (*20*). Cisplatin is also used in the treatment of ovarian,

[†] This work was supported by Grant CA086061 to J.M.E. and Grant CA55042 to L.D.S. from the National Institutes of Health. M.K. was partially supported by the Henry Bromfield Rogers Fellowship.

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[1] Abbreviations: 3-mA, 3-methyladenine; ϵA, 1,N^6-ethenoadenine; AAG, human 3-methyladenine DNA glycosylase; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi000417h CCC: $19.00
Published on Web 06/10/2000

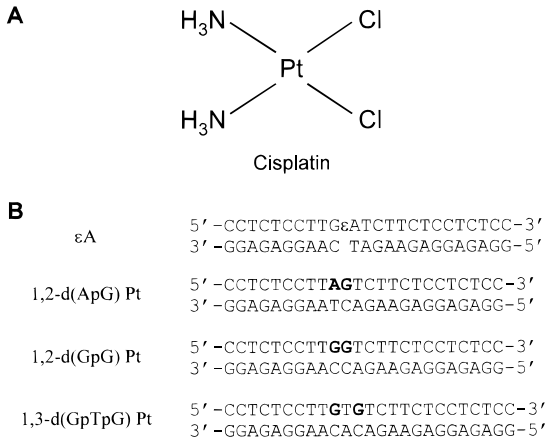


FIGURE 1: (A) Structure of cisplatin. (B) Sequences of the DNA duplexes used in the binding and glycosylase experiments. The pyrimidine-rich strand is designated as the top strand and the complementary strand is designated as the bottom strand. The bases involved in adduct formation are located in the top strand and are indicated in boldface type.

bladder, head and neck, and non-small-cell lung cancer (*19*), 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 (*21*). 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 cross-links and a small percentage of interstrand cross-links and monofunctional adducts (*22*−*25*).

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 (*17*−*19*). 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 (*26*−*29*). 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 (*30*, *31*) provide a structural signal for HMG box protein recognition (*32*, *33*).

The observation that DNA is bent when in complex with AAG and when it is coordinated by cisplatin (*11*, *31*, *34*), 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 (*34*) is reminiscent of the invasion by an amino acid residue of AAG into DNA containing a modified AP site (*11*). 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 cross-links, 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 nonproductively engaged in complexes with pseudosubstrates become significantly less effective in their role to defend cells against important DNA-damaging agents.

MATERIALS AND METHODS

Enzymes and Chemicals. The 5′-dimethoxytrityl ethenodeoxyadenosine 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 was from Sigma. The purified human AAG protein was a gift from Dr. Thomas Ellenberger (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA).

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 h, and platinated DNA was purified on denaturing polyacrylamide gels. The platination sites were confirmed by Maxam−Gilbert sequencing (*35*, *36*). The oligonucleotide containing ϵA was synthesized on an Applied Biosystems 391 automated DNA synthesizer by standard phosphoramidite chemistry. The ϵA oligonucleotide was deprotected in 10% DBU in methanol at room temperature for 48 h and purified by polyacrylamide gel electrophoresis (*37*). The sequences of the DNA duplexes are shown in Figure 1B. Concentrations were determined by measuring the A_{260} and calculating the extinction coefficients as described (*38*).

Gel Mobility Shift Assay. Binding assays were carried out in assay buffer containing 4 mM Tris-HCl 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 1 × TAE 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 ^{32}P-labeled, ϵA oligonucleotide. Amounts of bound and free ^{32}P-labeled probe were determined by quantitative analysis of dried gels on a Molecular Dynamics PhosphorImager. The equation used to determine the binding affinity of the protein to the different probes was

$$\Theta = \frac{1}{2T_t} + \left[K_t + \left(\frac{K_t}{K_c}\right)C_t + T_t + P_t - \left(\left[K_t + \left(\frac{K_t}{K_c}\right)C_t + T_t + P_t\right]^2 - 4T_tP_t\right)^{1/2}\right] \quad (1)$$

where Θ is the fraction of bound ^{32}P-labeled ϵA oligonucleotide, P_t, T_t, and C_t are the concentrations of the protein,

^{32}P-labeled ϵA oligonucleotide, and competitor DNA, respectively, and K_t and K_c represent the apparent dissociation constants for the ϵA and competitor DNA oligonucleotides, respectively (*39*, *40*). Competition curves were fit for the best value of K_c by nonlinear least-squares analysis. Relative DNA binding affinities were determined by comparing values of K_c 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-HCl buffer, pH 7.8, 100 mM KCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 1 mM EGTA, 80 nM−2.5 mM unlabeled competitor DNA, 0.7−1.1 nM ^{32}P-labeled DNA, and 11−14 nM human AAG. The DNA was chemically (0.1 N NaOH) or enzymatically (50 nM *Escherichia coli* formamidopyrimidine DNA glycosylase) 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 on a Molecular Dynamics PhosphorImager.

RESULTS

Human AAG Recognizes the Various Cisplatin Intrastrand Cross-Links. 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 cross-links by using a gel mobility shift assay. DNA duplexes containing each of the three intrastrand cross-links 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 cross-links as shown by the presence of a slower migrating band (Figure 2A, lanes 6, 8, and 10) not seen when these duplexes were analyzed in the absence of protein (Figure 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 2A, lane 2) as previously described (*5*−*7*). In contrast, little binding was observed when unmodified duplex DNA was used in the binding reaction (Figure 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).

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

Human AAG Binds Specifically to the Cisplatin Intrastrand Cross-Links. 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

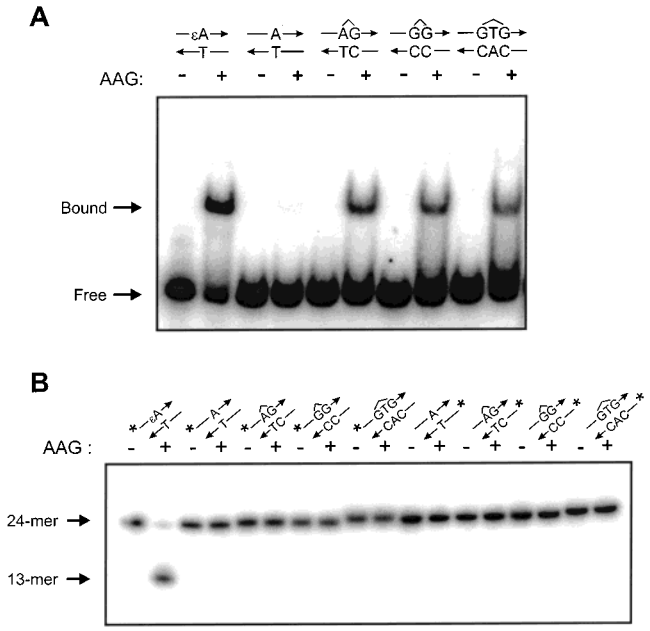
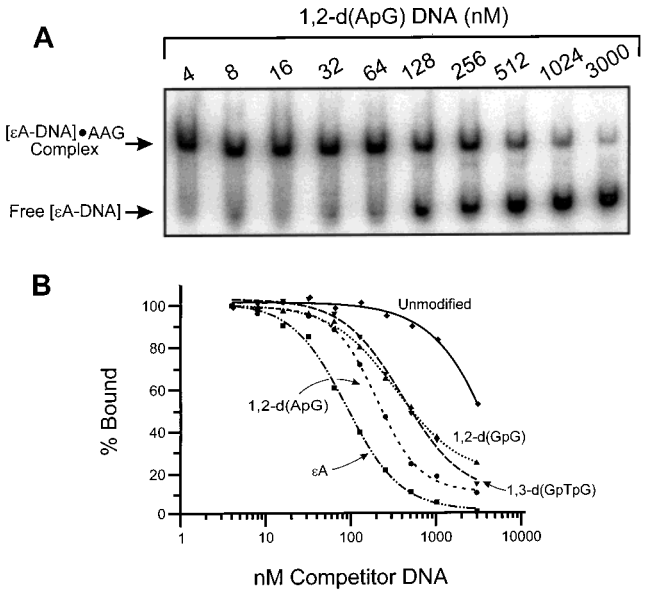


FIGURE 2: AAG recognizes the cisplatin intrastrand cross-links. (A) Gel mobility shift assay of human AAG with various oligonucleotide duplexes. The ^{32}P-labeled oligonucleotides containing a single ϵA (lanes 1 and 2), 1,2-d(ApG) (lanes 5 and 6), 1,2-d(GpG) (lanes 7 and 8), or 1,3-d(GpTpG) (lanes 9 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 mM 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).

showing the results of one such experiment is shown in Figure 3A. Addition of unlabeled 1,2-d(ApG) oligonucleotide competes away the binding of the human AAG protein to the ϵA oligonucleotide. Similar results were obtained with ϵA and the other cisplatin DNA adducts. A plot of the average of two experiments for each competitor DNA is shown in Figure 3B. The data were fit to a competitive binding equation (eq 1) and the apparent dissociation constants are presented in Figure 3C.

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 cross-links but not the 1,3-d(GpNpG) cross-link (*41*, *42*).

Interestingly, even though the two 1,2-intrastrand cross-links of cisplatin appear to bend and unwind the DNA helix to the same extent (*31*), the 1,2-d(ApG) cross-link is recognized more efficiently than the 1,2-d(GpG) cross-link, 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) cross-link is more distorted than the corresponding adducted guanine·cytosine base pair in the 1,2-d(GpG) cross-link (*43*). 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



C

DNA Duplex	Dissociation Constant [$K_{d(app)}$]
εA	26 nM
1,2-d(ApG)	71 nM
1,2-d(GpG)	115 nM
1,3-d(GpTpG)	144 nM

FIGURE 3: Specificity of AAG binding to cisplatin adducts. 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 purified human AAG protein and 0.2 nM ^{32}P-labeled ϵA duplex DNA. (B) 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 was present in the binding reaction. (C) DNA dissociation constants of the human AAG protein for each competitor DNA were determined from eq 1.

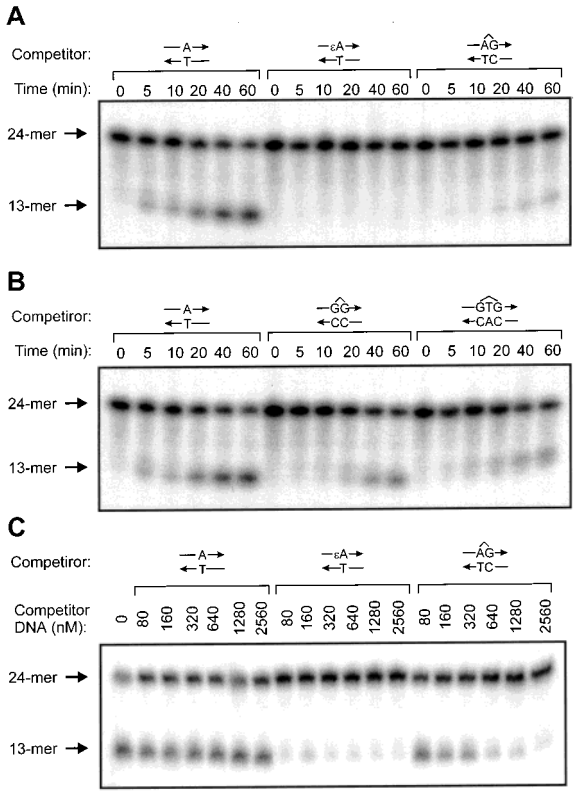


FIGURE 4: Inhibition of ϵA repair by cisplatin adducts. (A, B) ^{32}P-Labeled ϵA oligonucleotide was incubated with human AAG in the presence of 2500 nM unlabeled competitor DNA and removal of ϵA from the duplex DNA was measured over time. (A) Lanes 1−6, unmodified DNA; lanes 7−12, ϵA; lanes 13−18, 1,2-d(ApG). (B) Lanes 1−6, unmodified DNA; lanes 7−12, 1,2-d(GpG); lanes 13−18, 1,3-d(GpTpG). (C) ^{32}P-Labeled ϵA oligonucleotide was incubated with various concentrations of competitor DNA. Lane 1, no competitor DNA; lanes 2−7, unmodified DNA; lanes 8−13, ϵA; lanes 14−19, 1,2-d(ApG).

the *E. coli* RNA polymerase or the wheat germ polymerase II more effectively than the 1,2-d(ApG) adduct (*44*), and the 1,2-d(GpG) cross-link is more efficient than the 1,2-d(ApG) cross-link at inhibiting replication by DNA polymerases T7, Taq, and polymerase I (*45*), indicating that the polymerases can distinguish between the two structurally similar cross-links. Finally, the 1,2-d(ApG) cross-link is 4−5 times more mutagenic than the 1,2-d(GpG) cross-link (*46*), providing further evidence for this hypothesis.

Cisplatin Adducts Titrate AAG from Its Natural Substrates. On the basis of the high affinity of the human AAG protein for the cisplatin intrastrand cross-links, 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 ^{32}P-labeled ϵA oligonucleotide was incubated with 11 nM human AAG and 2500 nM 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 (Figure 4A,B lanes 1−6, and data not shown). In contrast, the presence of unlabeled ϵA (Figure 4A, lanes 7−12) or 1,2-d(ApG) (Figure 4A, 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 4B, lanes 7−12 and 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 4C, 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 4C, lanes 8−13). An increase in the AAG concentration resulted in some cleavage of the ^{32}P-labeled ϵA oligonucleotide when low concentrations of unlabeled ϵA oligonucleotide were present in the reactions (data not shown). When the 1,2-d(ApG) oligonucleotide was used 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 4C, 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 (data not shown). Furthermore, when

higher AAG concentrations were present in the reaction mixtures, higher concentrations of competitor DNA were required to inhibit the excision of ϵA to the same extent (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 2500-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/cell are typically observed in cisplatin-treated patients (*47*), whereas approximately 1−20 ϵA adducts/cell are detected in normal human liver tissue (*48*−*50*), suggesting that repair inhibition might also be operative in a clinically relevant context.

DISCUSSION

Cell survival is correlated with the formation and persistence of cisplatin adducts (*47*, *51*−*53*). Cells from tumor tissues that are generally more responsive to cisplatin chemotherapy appear to have lower levels of repair of cisplatin adducts (*53*−*55*). Therefore, the differential capacity for cisplatin adduct repair is postulated to be an important determinant of variability in clinical response to cisplatin therapy (*56*). 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 cross-links 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) cross-links 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.

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 nonhistone 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 (*34*). 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 (*34*). 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 (*11*). 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.

Ixr1 is a *S. cerevisiae* HMG box protein that also binds specifically to the 1,2-intrastrand cross-links of cisplatin (*57*). The dissociation constant for Ixr1 binding an oligonucleotide containing a single 1,2-d(GpG) is 250 nM (*58*). Furthermore, yeast strains deficient for Ixr1 are 2−6 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* (*59*). 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 (*59*). 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 5A). Interestingly, Northern analysis of polyadenylated RNA from different mouse tissues revealed that the highest Aag mRNA levels are seen in the testis (*3*). 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 (*60*, *61*). 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.

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 (*62*). 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 (*63*). There are, however, some in vitro data that support the notion that AAG may somehow enhance the repair of the 1,2-d(ApG) cross-link. One such study measured the relative rates of repair of the two 1,2-intrastrand cross-links by the mammalian excision nuclease. When excision repair is determined after incubation with crude human cell extracts, the 1,2-d(ApG) cross-link is a better substrate than the 1,2-d(GpG) cross-link. In contrast, when excision repair is measured after incubation with purified components of the mammalian excision repair machinery, the rates of repair of the two cross-links are indistinguishable (*64*). These results suggest that there might be a factor in the cell extracts that alters the rate of repair of one or both 1,2-intrastrand cross-links. Since the 1,2-d(ApG) adduct was recognized more efficiently by AAG than the 1,2-d(GpG)

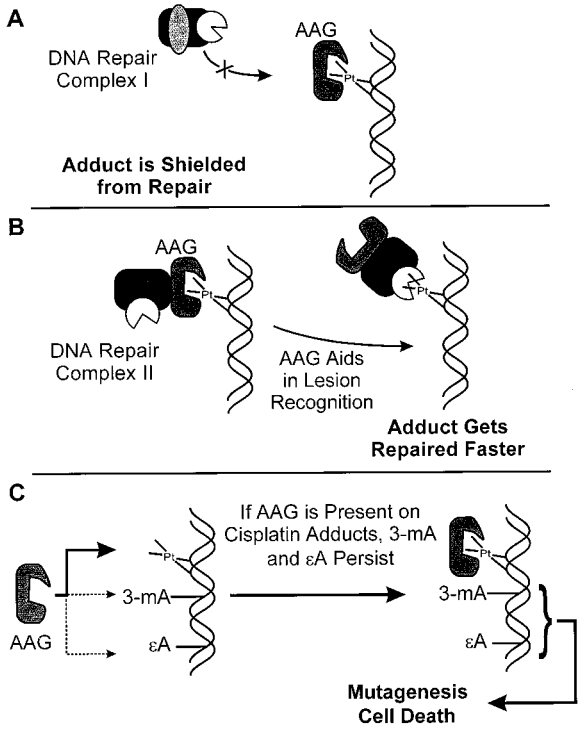


FIGURE 5: (A) Repair shielding model. Human AAG recognizes the cisplatin adducts and can inhibit their repair by physically blocking the access to other repair proteins. The cisplatin adducts persist in DNA, thereby potentiating their toxicity. (B) Stimulation of repair model. AAG recognizes cisplatin 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) 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.

adduct, it is possible that AAG might facilitate the rate of repair of the 1,2-d(ApG) cross-link by conveying this adduct to another repair system (Figure 5B).

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 (*65*, *66*). 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 multidrug chemotherapeutic regimens. Consistent with this hypothesis, cisplatin acts synergistically with several other drugs including BCNU (*67*).

ϵA arises in DNA as a result of lipid peroxidation or after exposure to ethyl carbamate or vinyl chloride (*68*, *69*). The latter compound is used in plastic production, and it has also been detected in BCNU-treated patients (*70*). 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 5C). 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 (*71*). Consequently, 3-mA is a toxic adduct that can have detrimental effects if left unrepaired (Figure 5C).

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 and mutagenicity 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.

ACKNOWLEDGMENT

We thank Dr. T. E. Ellenberger for providing human AAG and Drs. B. P. Engelward and M. D. Wyatt for helpful discussions.

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BI000417H