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Mutagenic and genotoxic effects of DNA adducts formed by the anticancer drug *cis*-diamminedichloroplatinum(II)

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ABSTRACT

The toxicity and mutagenicity of three DNA adducts formed by the anticancer drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) were investigated in *Escherichia coli*. The adducts studied were *cis*-[Pt(NH$_3$)$_2${d(GpG)}] (G*G*), *cis*-[Pt(NH$_3$)$_2${d(ApG)}] (A*G*) and *cis*-[Pt(NH$_3$)$_2${d(GpTpG)}] (G*TG*), which collectively represent ~95% of the DNA adducts reported to form when the drug damages DNA. Oligonucleotide 24-mers containing each adduct were positioned at a known site within the viral strand of single stranded M13mp7L2 bacteriophage DNA. Following transfection into *E.coli* DL7 cells, the genomes containing the G*G*, A*G* and G*TG* adducts had survival levels of 5.2 ± 1.2, 22 ± 2.6 and 14 ± 2.5% respectively, compared to unmodified genomes. Upon SOS induction, the survival of genomes containing the G*G* and A*G* adducts increased to 31 ± 5.4 and 32 ± 4.9% respectively. Survival of the genome containing the G*TG* adduct did not increase upon SOS induction. In SOS induced cells, the G*G* and A*G* adducts gave rise predominantly to G→T and A→T transversions respectively, targeted to the 5′ modified base. In addition, A→G transitions were detected for the A*G* adduct and low levels of tandem mutations at the 5′ modified base as well as the adjacent 5′ base were also observed for both adducts. The A*G* adduct was more mutagenic than the G*G* adduct, with a mutation frequency of 6% compared to 1.4% for the latter adduct. No *cis*-{Pt(NH$_3$)$_2$}$^{2+}$ intrastrand crosslink-specific mutations were observed for the G*TG* adduct.

INTRODUCTION

The great success of *cis*-diamminedichloroplatinum(II) (*cis*-DDP, Fig. 1) in the treatment of testicular cancer has resulted in the widespread application of this drug to combat many human malignancies (1). The therapeutic effects of *cis*-DDP result from its covalent binding to DNA to form a spectrum of adducts (2). The adducts are believed to contribute to the cytotoxicity of the drug by inhibiting replication and/or transcription. In addition to being genotoxic, DNA lesions formed by *cis*-DDP are also mutagenic. Since the mutagenicity of *cis*-DDP could play a role in the generation of second tumors in cancer patients, there is a need to understand the molecular basis for these genetic effects.

The most abundant DNA adducts formed by *cis*-DDP are *cis*-[Pt(NH$_3$)$_2${d(GpG)-*N*7(1),-*N*7(2)}] (G*G*, ~65%), *cis*-[Pt(NH$_3$)$_2${d(ApG)-*N*7(1),-*N*7(2)}] (A*G*, ~25%) and *cis*-[Pt(NH$_3$)$_2${d(GpXpG)-*N*7(1),-*N*7(3)}] [G*XG* (where X = A, C or T) ~5%] (3,4). The purpose of this study was to determine the relative genetic and toxic effects of each of these *cis*-DDP DNA adducts in isolation from the influence of the others by incorporating each adduct site specifically within a viral genome. By determining the relative contributions of each adduct to the total mutagenicity and genotoxicity of *cis*-DDP, it may be possible to identify those lesions that are principally genotoxic and those that are primarily premutagenic. This information could then be used for the design and evaluation of new platinum based drugs; ideally a new drug would maximize the number of genotoxic lesions and minimize the proportion of premutagenic lesions.

Since the therapeutic efficacy of *cis*-DDP is believed to result from the inhibition of DNA replication and transcription, a major goal of this work was to compare for the first time the relative ability of each adduct to inhibit DNA replication *in vivo*. The genotoxic potential of the G*G* adduct has been defined recently (5), but the relative inhibitory effects of the A*G* and G*XG* adducts are unknown. Somewhat more is known about the mutagenicity of the DNA adducts formed by *cis*-DDP; both the G*G* (5) and A*G* (6) adducts have been studied in *E.coli*. An important caveat, however, is that the A*G* adduct was studied in a pEMBL8(–) plasmid whereas the G*G* adduct was studied in an M13mp18 viral genome. Consequently, the results from the two systems are not strictly comparable. Furthermore, the mutagenicity of the G*XG* adduct has not been determined and early studies that used randomly modified DNA implicate G*XG* as a likely and frequent contributor to the mutational spectrum of cisplatin (7,8). Although the G*XG* adduct is less abundant than G*G* or A*G*, the possibility exists that it could be a major contributor to the overall mutagenicity of *cis*-DDP if it were disproportionately mutagenic compared to G*G* or

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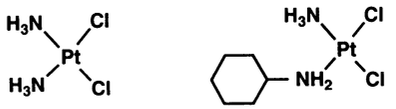

cis-DDP ACDP

Figure 1. Structures of platinum compounds.

A*G*. The present work reports both the genotoxic and mutagenic effects of all three of the major *cis*-DDP DNA intrastrand crosslinks determined in the same experimental system.

MATERIALS AND METHODS

Restriction endonucleases, T4 polynucleotide kinase and T4 DNA ligase were from New England Biolabs (the exceptions were *Stu*I and *Sca*I, which were from Boehringer-Mannheim). Phosphatases and DNAses used were from Sigma. The *E.coli* cell lines used were: DL7 [AB1157; lacΔU169, (9)] and GW5100 (JM103, P1$^-$, from G. Walker, MIT). M13mp7L2 bacteriophage genomes were a gift of C. W. Lawrence (University of Rochester). Sequencing reagents were from US Biochemical Corporation (Sequenase). [α-^{35}S]dATP was purchased from Amersham, [γ-^{32}P]ATP was purchased from New England Nuclear (DuPont). *cis*-DDP was from Sigma and purified as described (10). DNA purification systems were from the QIAGEN or Promega corporations. Oligonucleotides used in the construction of the singly *cis*-DDP modified genomes were synthesized by using Applied Biosystems reagents and a Model 381 DNA synthesizer.

Preparation of *cis*-DDP modified oligonucleotides

The oligonucleotide 24-mers 5′-d(TCT TCT TCT **AGG CCT** TCT TCT TCT), 5′-d(TCT TCT TCT CT**A GTA CTC** TCT TCT) and 5′-d(TCT TCT TCT **GTG CAC** TCT TCT TCT) were constructed to contain the *cis*-DDP G*G*, A*G* and G*TG* adducts respectively. The sites of platinum binding are underlined and the restriction endonuclease recognition sites *Stu*I, *Sca*I and *Apa*LI respectively, are shown in bold. These oligonucleotides were synthesized, purified, modified with *cis*-DDP and characterized by using previously described methods (11,12). The nomenclature used to refer to the oligonucleotide 24-mers is *Stu* 24, *Sca* 24 and *Apa*L 24 for the unmodified samples and *Stu* Pt, *Sca* Pt and *Apa*L Pt for their platinated counterparts. The presence of platinum on the modified oligonucleotides was verified by cyanide treatment to reverse the platinum adduct (13). The purity of the oligonucleotide 24-mers was determined by 5′ end labeling with [γ-^{32}P]ATP followed by denaturing polyacrylamide gel electrophoresis and quantitation by PhosphorImager analysis (Molecular Dynamics Corp.). The composition of the oligonucleotides was determined by digestion with DNAse I, nuclease P_1, alkaline phosphatase and calf intestinal phosphatase to reduce the DNA fragments to their constituent mononucleosides, which were analyzed by reversed phase HPLC (12). The sequence of the oligonucleotides was confirmed by DNA sequencing after incorporation into M13 single stranded (ss) genomes, as described below.

Preparation of *cis*-DDP modified genomes

The platinated, or unmodified, oligonucleotide 24-mers, described above, were incorporated into ss M13 genomes by using described methodology (14,15). Briefly, ss M13mp7L2 DNA was digested with *Eco*RI to remove its 42 nucleotide hairpin structure, thereby creating a linearized ss genome. Linearized ss M13mp7L2 DNA was separately annealed to one of three different scaffold oligonucleotide 64-mers. The ends of the scaffold oligonucleotides contained sequences complementary to 20 bases on both the 5′ and 3′ ends of the linearized M13mp7L2 genome while the centers of each of the three different scaffold oligonucleotides formed 24 base 'gaps' complementary to the *Stu* 24 and *Stu* Pt, *Sca* 24 and *Sca* Pt or *Apa*L 24 and *Apa*L Pt oligonucleotide 24-mers respectively. The oligonucleotide 24-mers were 5' phosphorylated by using T4 polynucleotide kinase, annealed to their respective complementary gap sequences and covalently ligated into the M13mp7L2 genome by using T4 DNA ligase. In parallel, control genomes were constructed without any 24-mer present to ensure that ligation across the 24 base gap did not occur. Genomes incorporating each of the six oligonucleotide 24-mers were constructed in triplicate.

The singly *cis*-DDP modified ss M13mp7L2 genomes were characterized by heat denaturation to remove the scaffold oligonucleotide. Heating at 90°C for 2 min removed the scaffold oligonucleotides, resulting in linear ss genomes if an oligonucleotide 24-mer had not ligated into its complementary 'gap' or if the oligonucleotide had been covalently attached at only its 5′ or 3′ end. Such linear ss M13 genomes were biologically inactive (data not shown). Conversely, if an oligonucleotide 24-mer had been ligated at both its 5′ and 3′ termini, a completely covalently closed genome was formed that remained circular after heat treatment. Circular genomes were distinguished from the incompletely ligated, linear genomes by agarose gel electrophoresis to provide an estimate of the ligation efficiency.

Determination of the genotoxicities of G*G*, A*G* and G*TG* adducts

Toxicities of the G*G*, A*G* and G*TG* adducts were determined by electroporating the ss M13 genomes containing the *Stu* Pt, *Sca* Pt and *Apa*L Pt oligonucleotides respectively, into *E.coli* DL7 cells in parallel with the unmodified *Stu* 24, *Sca* 24 and *Apa*L 24 control genomes. Survival was determined by comparing the relative number of infective centers formed by *cis*-DDP modified genomes to that of the unplatinated genomes. Genomes were electroporated into *E.coli* DL7 cells that had, or had not, been induced for the SOS response. Induction of the host SOS response by UV irradiation and electroporations were both carried out as previously described (5,16). Immediately before electroporation, 40 ng of each genome, determined by A_{260} OD readings, was diluted to 100 μl in water and heated as described above to remove the scaffold oligonucleotide. One hundred microliters of cells was added to the DNA and the resulting mixture was electroporated. Transformation mixtures were plated immediately and survival values, representing the genotoxicity of each adduct, were based on the production of infective centers

(5). Progeny phage were grown and processed as described below to determine the mutagenicity of each adduct.

Determination of the mutagenicities of G*G*, A*G* and G*TG* adducts

Progeny phage were combined from 23–24 replicate transfections of each of the six genomes studied (containing the *Stu* 24, *Stu* Pt, *Sca* 24, *Sca* Pt, *Apa*L 24 or *Apa*L Pt oligonucleotides) into SOS uninduced cells to give a pool of ~1×10^6 phage. Similarly, approximately the same number of phage was pooled from 41–42 replicate transfections into SOS induced cells. Mutational analysis of these progeny phage was based on the strategy described by Klein *et al.* (17). Briefly, the pooled progeny phage were used to produce small scale replicative form (RF) DNA preparations. The RF DNA was digested with the restriction endonucleases *Stu*I, *Sca*I or *Apa*LI, as appropriate. Mutations within a restriction site render the site refractory to digestion. Digestion of the RF DNA, therefore, linearizes the wild type genomes and enriches for genomes containing mutations within the *Stu*I, *Sca*I or *Apa*LI sites. Such mutations presumably arise from the G*G*, A*G* or G*TG* adducts respectively. The digested DNAs were transfected into *E.coli* DL7 cells in parallel with equal amounts of undigested DNAs. Only mutant DNAs in the digested samples should remain viable and produce infective center plaques, whereas the DNAs in the undigested samples should be 100% viable. The mutation frequencies, determined by comparison of the relative number of plaques from the digested samples to the undigested samples, include all mutations that occurred within the six base restriction recognition site. Mutations specifically attributable to *cis*-$\{Pt(NH_3)_2\}^{2+}$ intrastrand crosslinks were determined by DNA sequence analysis (*vide infra*).

RESULTS

Preparation of modified oligonucleotides

Oligonucleotides containing the G*G*, A*G* and G*TG* *cis*-$\{PT(NH_3)_2\}^{2+}$ intrastrand crosslinks, as well as their respective unmodified counterparts, were characterized after synthesis and purification. Base composition analysis and DNA sequencing showed that each of the unmodified oligonucleotides had the expected base composition and sequence. The purity of the unmodified oligonucleotides was determined to be >99.7% by 5′ end labeling with [γ-^{32}P]ATP followed by denaturing polyacrylamide gel electrophoresis and PhosphorImager quantitation. By this technique, the modified oligonucleotides containing the G*G*, A*G* and G*TG* adducts were >99.7, 99.4 and 98% homogenous respectively. Nucleoside digestion analysis demonstrated that the *cis*-DDP lesion had formed at the desired d(GpG), d(ApG) or d(GpTpG) sequence in the respective oligonucleotides.

Preparation of *cis*-DDP modified genomes

Bacteriophage ss M13 genomes containing the G*G*, A*G* and G*TG* adducts were constructed by a modification of a procedure originally used to construct genomes containing UV-DNA lesions (18). Scaffold oligonucleotide 64-mers were purified to >95%. Annealing of the scaffold oligonucleotides to linearized ss genomes converted ~50% of the material to a circular form based on analysis of ethidium bromide stained agarose gels. Circular constructs contained ss gaps complementary to the oligonucleotide 24-mers. Each of the six oligonucleotide 24-mers ligated into their respective complementary sequences with high efficiency as evidenced by the nearly complete conversion of the DNA to a covalently closed (non-heat denaturable) circular ss form. By contrast, for samples that were subjected to ligation conditions, but in the absence of 24-mer, all of the originally circular material was converted back to the linear form after heat denaturation. This control established that the 5′ and 3′ ends of the linearized M13mp7L2 genomes did not religate. In an additional control experiment, fully annealed (i.e. in the presence of 24-mer) but unligated genomes were subjected to heat denaturation. Unlike samples that underwent ligation, these genomes were also converted to linear form by heat, indicating that the heat denaturation process efficiently removed the scaffold oligonucleotide. Additional evidence that the scaffold was adequately removed by the heat denaturation process is derived from the *in vivo* results for the G*G* adduct. The G*G* adduct has previously been studied in both ss and ds DNA contexts (5). The results of the present work, detailed below, closely conform to the results previously observed for this adduct in ss DNA, thus supporting the conclusion that the scaffold oligonucleotide had been removed.

A test transformation demonstrated that linear ss M13 genomes were biologically inactive and therefore incapable of producing progeny in *E.coli* DL7 cells. Portions of the ligation reactions done in the absence of 24-mer, as well as fully annealed but unligated material, were linearized by heat denaturation to remove the scaffold and transfected into *E.coli* DL7 cells in parallel with circular genomes constructed with unmodified 24-mer. The linearized ss M13 genomes had a plaque forming ability at least 10^5-fold lower than comparable circular genomes. This result demonstrated that the *cis*-$\{Pt(NH_3)_2\}^{2+}$ intrastrand crosslink modified ss M13 genomes could be used without further purification, since only the desired product, a circular genome with a 24-mer covalently ligated at both ends, was viable.

Genotoxicities of the G*G*, A*G* and G*TG* adducts

The genotoxicities of the *cis*-DDP DNA adducts are graphically summarized in Figure 2. In SOS uninduced *E.coli* DL7 cells, the G*G* adduct was the most toxic lesion with a survival of only 5.2 ± 1.2% compared to unmodified genomes. Genomes containing the G*TG* adduct had a 2.5-fold higher survival at 14 ± 2.5% whereas survival for A*G* containing genomes was even higher at 22 ± 2.6%. Upon SOS induction, survival of genomes with G*G* and A*G* adducts increased to about the same absolute level, 31 ± 5.4 and 32 ± 4.9% respectively. The relative increase in survival, however, was much higher for the G*G* adduct (~6-fold) than for the A*G* adduct (~1.5-fold). Interestingly, survival was not enhanced upon SOS induction for the G*TG* adduct, remaining at 15 ± 3.7%, a value not significantly above the 14 ± 2.5% survival value for SOS uninduced cells.

Mutagenicities of the G*G*, A*G* and G*TG* adducts

The mutation frequencies of the G*G*, A*G* or G*TG* adducts situated in ss M13 genomes replicated in either SOS induced or SOS uninduced *E.coli* DL7 cells were determined in parallel with unmodified control genomes. Mutation frequencies are listed in Table 1. Only samples that contained a single (or in some cases tandem) base change within the restriction sites were scored as

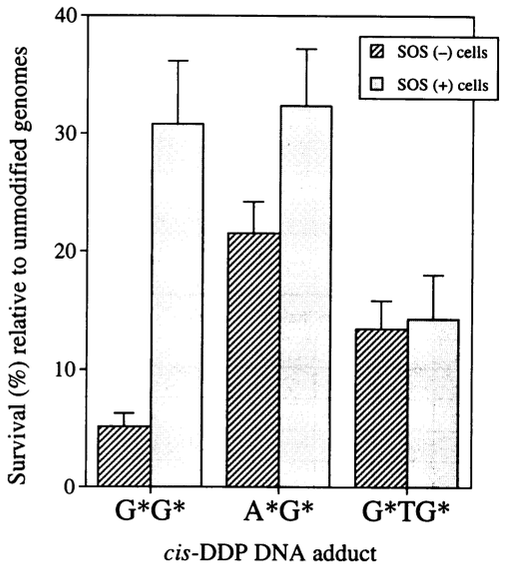

Figure 2. Survival of singly *cis*-DDP modified M13 ss genomes in SOS induced or non induced, *E.coli* DL7 cells. Survival values were determined by comparing the relative number of infective center plaques formed by *cis*-DDP modified genomes to that of unmodified control genomes. The data from the non SOS induced cells are mean values for 23–24 replicas; the data from the SOS induced samples represent 40–42 replicas.

putative *cis*-DDP mutants. The type and distribution of mutations in the *Stu*I (G*G*), *Sca*I (A*G*) or *Apa*LI (G*TG*) restriction sites are given in Table 2. The mutations from unmodified genomes replicated in SOS induced or uninduced cells, or from platinated genomes replicated in SOS uninduced cells, were randomly distributed (Table 2a, b or c, bottom three panels). These mutations did not occur more frequently at the bases modified with platinum than at other bases within the restriction sites. By contrast, genomes containing either G*G* or A*G* adducts that had been replicated in SOS induced cells experienced a significant enhancement of specific mutations at the sites of platinum modification (Table 2a and b, top panel). For example, 75% of the 81 mutants identified in the G*G* spectrum (Table 2a) were G→T transversions at the 5′ dG of the d(AGGCCT) sequence, suggesting that these mutations were a consequence of *cis*-$\{Pt(NH_3)_2\}^{2+}$ intrastrand crosslink modification. Additionally, G*G* induced tandem d(ApG) to d(TpT) and d(CpT) mutations were observed, albeit at a much lower frequency. The combined G*G* induced mutations occurred at a frequency of 1.4% (Table 3). This value is slightly lower than the 'adjusted' mutation frequency of Table 1 because only mutations targeted at either of the two bases of the adduct were included; as can be seen from Table 2, most mutations were targeted.

A*G* mutations were also highly targeted, with 65% of the 100 mutants identified consisting of A→T transversions at the 5′ base of the A*G* adduct. A tandem d(TpA)→d(CpT) double mutation occurred less frequently. In addition to the more prevalent A→T transversion, A→G transitions also were induced by the A*G* adduct (Table 2b). Overall, the A*G* adduct had a mutation frequency of 6% (Table 3).

In comparison to the A*G* and G*G* adducts, the G*TG* adduct produced no targeted mutations, even in SOS induced cells. As shown in Table 2c, the most abundant mutation, 23% of the total 143 samples analyzed, was a C→T transition at the 5′ dC of the d(GTGCAC) sequence. This mutation, however, was comparably abundant (at 29%) in the unmodified spectrum indicating that it was not drug induced. Furthermore, mutations did not occur more frequently at the *cis*-DDP modified dGs than at other bases within the *Apa*LI restriction site. These data indicate that the G*TG* adduct, at least in our system, was not mutagenic in *E.coli* under cellular conditions that rendered the G*G* and A*G* adducts mutagenic.

Table 1. Mutation frequencies and genetic requirements for mutagenesis by platinum–DNA adducts

Restriction site[a]	*cis*-DDP modified	SOS induced	RRF_1[b]	RRF_2[c]	Mutation frequency[d]
AGGCCT	+	+	5.1×10^{-2}	11/32	1.7%
(*Stu*I)	–	+	1.4×10^{-2}	8/32	0.35%
	+	–	0.75×10^{-2}	19/56	0.25%
	–	–	0.12×10^{-2}	15/38	0.05%
AGTACT	+	+	9.1×10^{-2}	24/32	6.8 %
(*Sca*I)	–	+	1.3×10^{-2}	2/32	0.08%
	+	–	1.3×10^{-2}	5/32	0.20%
	–	–	1.2×10^{-2}	0/30	<0.04%
GTGCAC	+	+	4.1×10^{-2}	41/128	1.3 %
(*Apa*LI)	–	+	2.9×10^{-2}	4/32	0.36%
	+	–	0.89×10^{-2}	25/32	0.70%
	–	–	0.88×10^{-2}	26/32	0.72%

[a]The restriction recognition sites containing the three *cis*-DDP DNA adducts (underlined) are shown.

[b]The RRF_1 (restriction resistant fraction) is the fraction of the progeny phage pool that scores as resistant to one treatment with the restriction endonuclease indicated in parenthesis. This treatment selects for targeted adduct induced mutants and reduces, but does not eliminate, the wild type offspring. In addition to wild type contamination, the RRF_1 includes M13mp7L2 genomes from incomplete *Eco*RI digestion during genome construction and a collection of 'genetic engineering' mutations; the latter consist of large (~800 bp) deletions, smaller (~30–80 bp) deletions generally lacking the entire sequence corresponding to the 24-mer and varying lengths of adjacent DNA and a specific 15 bp deletion consisting of the restriction site and one of the flanking d(TCT TCT TCT) sequences. The 'genetic engineering' mutations collectively accounted for <0.1% of all progeny.

[c]DNA sequencing of independent RRF_1 clones revealed the fraction of sequences within that pool with point mutations targeted to the restriction site containing the adduct; this fraction is defined as RRF_2.

[d]The mutation frequency of the adduct, defined as all single and double mutations within the denoted restriction site, is $(RRF_1)(RRF_2)(10^2)$.

DISCUSSION

This study has examined the relationship between structures and biological activities of the three major DNA adducts formed by *cis*-DDP. The data obtained are consistent with and extend a developing model that explains the molecular effects of *cis*-DDP upon DNA. Separate studies by us and others have previously partially examined the biological properties of two of the three most abundant *cis*-DDP adducts, but not in the same experimental system. Specifically, Fuchs and coworkers studied the muta-

Table 2a: Relative abundance of mutations (in %) induced by the G*G* *cis*-DDP adduct [a]

SOS Induced — Platinated (n = 81)

	T	A	G	G	C	C	T
A		—	4.9	1.2		1.2	
C	1.2	2.5		2.5	—	—	
G			—	—			
T	—	3.7	75.0	2.5			—
Δ							1.2
other [b]		T(C) – T 2.5			ΔCCT 1.2		

SOS Induced — Unplatinated (n = 63)

A		—	6.4	3.2		4.8	4.8
C	4.8	9.5	1.6	3.2	—	—	4.8
G		1.6	—	—	4.8	4.8	
T	—	4.8	4.8	7.9	4.8	4.8	—
Δ		3.2	9.5		9.5		1.6
other							

SOS Uninduced — Platinated (n = 19)

A		—	16.0	5.3			
C		32.0	21.0		—	—	
G			—	—	5.3		
T	—	5.3	11.0				—
Δ					5.3		
other							

SOS Uninduced — Unplatinated (n = 58)

A		—	5.2	1.7	1.7	1.7	
C		17.0	5.2	6.9	—	—	1.7
G		1.7	—	—	3.5	1.7	
T	—	6.9	5.2		3.5	8.6	—
Δ		5.2	8.6		12.0		
other						+C 1.7	

[a]The target DNA sequence, with or without *cis*-DDP modification at the underlined bases, is given in large type across the top of the table. Mutations observed in this DNA sequence after replication in SOS induced or non-induced cells are indicated in the boxes below the sequence. The numbers given are the relative proportion of each mutation observed, given as a percentage of *n* (where *n* = the total number of mutations determined for each panel). A horizontal line denotes that no mutation is possible (e.g. G→G), whereas no data within a box indicate that no mutation was observed.

[b]Two double mutations d(ApG)→d(TpT) and d(ApG)→d(CpT) both occurred at a frequency of 2.5%.

Table 2b: Relative abundance of mutations (in %) induced by the A*G* *cis*-DDP adduct [a]

SOS Induced — Platinated (n = 100)

	T	A	G	T	A	C	T
A		—	1.0		—		1.0
C				4.0		—	1.0
G		16.0	—				
T	—	65.0		—		4.0	—
Δ						1.0	
other	C–T 7.0						

SOS Induced — Unplatinated (n = 51)

A		—	14.0	3.9		2.0	5.9
C		5.9		18.0	5.9	—	3.9
G		5.9	—		3.9		2.0
T	—	14.0		—	2.0		—
Δ		5.9					2.0
insertion				2.0	2.0		

SOS Uninduced — Platinated (n = 38)

A		—	7.9		—	7.9	
C			5.3	7.9		—	11.0
G			—				
T	—	5.3		—		7.9	—
Δ		11.0		2.6	7.9	5.3	18.0
other							

SOS Uninduced — Unplatinated (n = 7)

A		—	29.0		—		
C		14.0		29.0		—	
G			—				
T	—			—			—
Δ					29.0		
other							

[a]See Table 2a.

genicity of the A*G* adduct situated at a specific site in ds DNA (6) and our laboratory previously studied the G*G* adduct in both ss and ds DNA. Both A*G* and G*G* induced transversions asymmetrically located at the 5′ modified base (5,6). These independent studies also revealed a strict SOS dependence for mutagenicity. The present study has shown that the A*G* adduct is 4–5-fold more mutagenic than the quantitatively more abundant G*G* adduct, when the two adducts are compared in the same experimental system. No data previously existed with regard to the mutagenic capability of the G*XG* adduct, although indirect evidence from one group (7,19) suggests that this lesion may be a major contributor to the mutational spectrum of *cis*-DDP. Our results undercut that hypothesis in that the G*TG* adduct was not detectably mutagenic. A second major goal of this work was to compare for the first time the relative ability of each adduct to inhibit DNA replication *in vivo*. This area is of interest owing to the widespread use of *cis*-DDP in anticancer regimens. The G*G* adduct was the most potently lethal of the three studied, although its lethality could be suppressed by 6-fold upon induction of the DNA repair- and replication-enhancing SOS functions of the host. The A*G* adduct was less toxic than the G*G* adduct. Interestingly, the toxicity differential between the G*G* and A*G* adducts disappeared under conditions of SOS induction. The G*TG* adduct of *cis*-DDP showed an intermediate level of toxicity, which was unaffected by induction of the SOS system.

One noteworthy feature of the results is the observation of mutations from G*G* and A*G* primarily at the DNA base of the dinucleotide adduct showing the maximal structural distortion. An important caveat is that structural studies have emphasized the effects of adducts on the architecture of duplex DNA. The present genetic studies, for reasons explained below, were done on adducts introduced in single stranded form into cells. In the absence of information on adduct induced structural effects on ss DNA, we use the body of information on duplex structures as a framework within which to construct hypotheses. In this study, mutations induced by the G*G* and A*G* adducts were targeted with high specificity to the 5′ base of the adduct. This result is consistent with structural information discussed above that the 5′ base of a *cis*-DDP modified dinucleotide sequence suffers the

Table 2c: Relative abundance of mutations (in %) induced by the G*TG* *cis*-DDP adduct [a]

SOS Induced — Platinated (n = 143)

	T	G	T	G	C	A	C
A		9.8	2.1	5.6	7.7	—	0.7
C		3.5	1.4	3.5	—	7.0	—
G		—	2.1	—	0.7	4.2	0.7
T	—	1.4	—	2.1	23.0	1.4	4.2
Δ		0.7	4.2	2.8	0.7	0.7	
other [c]				7.0 T–T 2.8			

SOS Induced — Unplatinated (n = 104)

A		5.8	2.9	5.8	1.0	—	1.0
C		3.9	1.0	3.9	—	13.0	—
G		—	1.0	—	2.9	1.9	
T	—	3.9	—	1.9	29.0	1.0	12.0
Δ		1.0	1.9	1.0	1.0	1.0	
other				T–G 0.96			

SOS Uninduced — Platinated (n = 25)

A		32.0		12.0	4.0	—	
C				4.0	—	8.0	—
G		—		—			
T	—	4.0	—				20.0
Δ			4.0	4.0	4.0		
other							

SOS Uninduced — Unplatinated (n = 26)

A		19.0		3.9		—	
C			7.7	12.0	—	3.9	—
G		—		—			
T	—		—		27.0		23.0
Δ				3.9			
other							

[a]See Table 2a.

[c]Both the d(GpC) and d(CpA) sequences were mutated to d(TpT) at the indicated frequencies.

Table 3.

A G*G* C C T		T A*G* T A C		G* T G* C A C
↓		↓		↓
T	1.3 %	T	4.4 %	No *cis*-DDP induced
T-T	0.04 %	G	1.1 %	mutants observed
C-T	0.04 %	C-T	0.5 %	
Total:	**1.4 %**		**6.0 %**	

[a]Mutations were determined to be *cis*-DDP induced by analysis of the mutational spectra presented in Table 2. Mutations that occurred at a significantly higher frequency at or near the site of platinum modification in the SOS(+) *cis*-DDP modified samples compared to the unmodified and SOS(–) samples were deemed to be drug induced. The numbers shown here are slightly smaller than those for the same adducts in Table 1, because only mutations targeted specifically to the adducted bases (see Table 2) were included in these calculations.

greatest distortion upon platinum binding. By contrast, the 3′ modified G is relatively unperturbed from its normal B-DNA conformation and, as the data show, did not experience drug induced mutations. Similarly, the mutations induced by the thymine–thymine cyclobutane dimer formed by UV light are asymmetrically localized to the nucleotide showing the most deviation from normal structure. In this case, the DNA is most severely distorted at the 3′ modified base (20). Accordingly, mutations arising from thymine dimers are highly targeted to the 3′ modified base, with ≥90% of all induced mutations occurring at this site (15,18).

The structure of the A*G* adduct has not been as extensively characterized as the G*G* adduct. Nevertheless, it appears that the nature of the structural perturbations imposed on DNA by both adducts is similar (21,22), thereby accounting for the similar mutational specificities of each lesion. The extent of structural distortion caused by binding at d(ApG) sites, however, is greater than that caused by binding to d(GpG) sites (23–25). The greater structural distortion imposed by *cis*-DDP binding at d(ApG) sites compared to d(GpG) sites, could account for the 4-fold higher mutagenicity of the former lesion.

The results of the present study are in accord with most of the literature on platinum mutagenesis. There has been a single report of mutations preferentially occurring at the 3′, rather than the 5′, modified base (26) of a putative platinum crosslink. Most of the literature, however, is in agreement with the structural argument presented above that mutations occur primarily at the nucleotide showing the most structural distortion. For example, the types of mutations induced by the G*G* and A*G* adducts (Table 3) in the current study are almost identical to those previously attributed the same adducts in other systems (5,6,16,27,28). Furthermore, the 5–10-fold higher mutagenicity of the A*G* adduct, predicted previously on the basis of indirect evidence, is formally established in the current study in which the A*G* adduct (6%) was 4.3-fold more mutagenic than the G*G* adduct (1.4%). The absolute mutation frequencies observed here were much higher than the 0.2 and 1–2% previously seen for the G*G* and A*G* adducts respectively (5,6). In the previously published studies, however, the adducts were situated in duplex genomes susceptible to UvrABC repair or strand bias effects; the latter cause asymmetric replication in favor of the unmodified DNA strand. The current experiments used ss DNA, which is refractory to such effects, thereby allowing the *cis*-$\{Pt(NH_3)_2\}^{2+}$ intrastrand crosslinks to exhibit quantitatively higher mutagenicities.

In conclusion, the present results afford significant insights into the genetic effects of DNA adducts formed by *cis*-DDP that can be exploited in the design and evaluation of other platinum based anticancer drugs. In particular, the G*G* adduct is emerging as an ideal candidate for inclusion in the DNA binding spectrum of a platinum drug (16). The present study has demonstrated that the G*G* adduct is the most genotoxic of the three major *cis*-$\{Pt(NH_3)_2\}^{2+}$ intrastrand crosslinks, which collectively account for ~95% of the adducts formed by *cis*-DDP (3,29). Although highly toxic, the G*G* adduct is ~4–5-fold less mutagenic than the A*G* adduct. The mutagenic properties of any drug widely used in humans are important factors to consider since mutagenicity may lead to the development of second tumors. In this regard, drugs with a high ratio of toxicity to mutagenicity are desirable. This study suggests that platinum drugs be designed to maximize the occurrence of the G*G* adduct and minimize the frequency of the more mutagenic A*G* adduct. The practical utility of this approach is manifested in the genetic effects of *cis*-ammine(cyclohexylamine)dichloroplatinum(II) (ACDP, Fig. 1). ACDP is the biologically active metabolite of a therapeutically promising platinum(IV) compound (30). ACDP binds to DNA to form a spectrum of adducts

similar to *cis*-DDP, with the notable exception that its cyclohexyl ring directs formation of 3-fold fewer A*G* adducts (8%) compared to *cis*-DDP (~25%) (31). A recent analysis of the genetic effects of *cis*-DDP and ACDP demonstrated that, whereas both drugs were equally genotoxic, *cis*-DDP was 2-fold more mutagenic than ACDP (16). This result is consistent with the higher proportion of the highly mutagenic A*G* adduct occurring in the *cis*-DDP DNA binding spectrum.

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