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Base-pair substitution hotspots in GAG and GCG nucleotide sequences in *Escherichia coli* K-12 induced by *cis*-diamminedichloroplatinum (II)

(forward mutagenesis/LacI system/antitumor activity/DNA repair/DNA crosslinks)

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ABSTRACT **Cell killing and mutation induction by *cis*- and *trans*-Pt$(NH_3)_2Cl_2$ in *Escherichia coli* were examined by studying forward mutagenesis in the *lacI* gene in cells with different repair capacities. Survival experiments showed that repair-proficient cells were slightly more sensitive for the *cis* isomer than for the *trans* isomer, whereas repair-deficient RecA and UvrB cells were extremely sensitive only for the *cis* compound. *cis*-Pt$(NH_3)_2Cl_2$ induced mutagenesis in both wild-type cells and RecA cells but not in UvrB cells; whereas no detectable mutagenesis was induced by treatment with the *trans* compound. Examination of the nature of the mutations induced by *cis*-Pt$(NH_3)_2Cl_2$, by using the LacI system, revealed that base-pair substitutions leading to nonsense mutants are only induced in wild-type cells, suggesting that the intact products of both the *uvrB* and the *recA* gene are necessary for the repair responsible for this type of mutagenesis. Investigation of the nonsense mutants reveals that 70% of these mutations result from GC → TA or GC → AT substitutions at sites where the guanine is part of a GAG or GCG sequence. These results are discussed in relation to existing theories on the interaction between Pt compounds and DNA. A model for Pt–DNA adducts, leading to base-pair substitutions, is proposed.**

About a decade ago, Rosenberg *et al.* (1) reported that *cis*-Pt$(NH_3)_2Cl_2$ shows antitumor activity against sarcoma 180 and leukemia L1210, whereas *trans*-Pt$(NH_3)_2Cl_2$ is ineffective. Since then it has been shown that several other *cis* Pt(II) and *cis* Pt(IV) compounds exhibit antitumor activity (2, 3). In mammalian, as well as in bacterial cells, DNA is the preferential target for Pt compounds. For *cis*-Pt$(NH_3)_2Cl_2$ this interaction results in lesions that selectively block DNA replication (4, 5). In this respect, *cis*-Pt compounds behave similar to other drugs such as alkylating and radiomimetic agents.

In vitro, *cis*-Pt$(NH_3)_2Cl_2$ binds to bases in DNA, and the order of binding affinity has been shown to be guanine>adenine>cytosine≫thymine, with a strong preference for the N-7 position of guanine (6). Monofunctional binding to a single base is unlikely to be the principal lesion through which *cis*-Pt$(NH_3)_2Cl_2$ exerts its antitumor activity because, at equitoxic doses, more of the inactive *trans* compound is bound to DNA (7). Therefore, specific bifunctional binding of *cis*-Pt$(NH_3)_2Cl_2$ to DNA is thought to be responsible for its antitumor activity.

For the bifunctional mode of action, several models have been proposed such as intrabase chelation at the O-6 and N-7 positions of guanine (6,8), interstrand crosslinking between the N-7 positions of guanines in opposite strands (3, 7, 9), and intrastrand crosslinks between two, presumably adjacent, guanines in the same strand (10–12).

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In bacteria (13–17), as well as in eukaryotic cells (18–20), a correlation between mutagenicity and antitumor activity of several Pt compounds has been found, suggesting that lesions leading to mutation events can also be responsible for antitumor activity. Several indications suggest that, in prokaryotic cells, repair of Pt-induced lesions can occur through excision and recombination (21). In eukaryotic cells the involvement of excision repair has been reported (22, 23).

This paper presents results of an investigation on the nature of *cis*-Pt$(NH_3)_2Cl_2$-induced mutations in *Escherichia coli* cells with different repair capacities, using the LacI system developed by Miller and coworkers (24–26). In this system, forward mutations in the *lacI* gene situated on a F′ episome are selected. By conjugation of the F′ episomes carrying the *lacI* mutations into several suppressor strains, it can be determined which of the induced mutations are nonsense mutations. Subsequently, the positions of the nonsense mutations in the *lacI* gene are determined by conjugation of the F′ into strains carrying *lacI* deletions of various lengths. Dependent on the position of the mutations, $LacI^+$ recombinants can be formed or not. In this way it is possible to detect 72 independent transition and transversion events, which lead to amber (TAG) and ocher (TAA) codons, in the known sequence of the *lacI* gene of *E. coli*. Determination of sites where nonsense mutations are preferentially induced (hot spots) may provide information about the interaction of the mutagenic agent with a certain nucleotide sequence.

MATERIALS AND METHODS

Treatment of the Cells with Pt Compounds. Exponentially growing cells in tryptone broth were harvested, resuspended in Vogel–Bonner buffer, and incubated for 60 min at 37°C. Subsequently, freshly prepared solutions of *cis*- and *trans*-Pt$(NH_3)_2Cl_2$ in dimethylformamide were added. The amount of dimethylformamide in the cell suspension never exceeded 1% of the total volume. The cells were incubated with the Pt compounds for 120 min at 37°C in the dark, spun down, and washed with ice-cold buffer.

Survival and Mutagenesis. After treatment with Pt compounds, the cells were chilled on ice and dilutions were plated with 4 ml of soft agar on LC plates to determine survival and on phenyl β-D-galactoside plates to score LacI mutants (24). The spontaneous frequency of LacI mutants depends on the number of cells present per plate. Therefore, spontaneous frequencies were determined at various cell concentrations, in order to calculate the number of mutants induced upon treatment with the Pt compounds.

LacI System. Materials and techniques used in the LacI system were as described by Miller and coworkers (24–26), with

the exception that the cells were plated directly after the mutagenic treatment.

Media. The tryptone-broth (LC) medium contained 1% Difco tryptone and 0.5% Difco yeast extract supplemented with 0.8% NaCl. The minimal medium (Vogel–Bonner) has been described (27). Minimal medium was supplemented, when required, with amino acids at 50 μg/ml, thiamin at 5 μg/ml, biotin at 0.5 μg/ml, and glucose to a final concentration of 1%. Unsupplemented minimal medium was used as a buffer for washing and diluting. For solid media, 1.5% Difco Bacto agar was added; half this concentration was used for soft agar. The other media used in the LacI system have been described by Miller *et al.* (24–26).

Strains. *E. coli* K-12 strains carrying deletions in the *lacI* gene and the suppressor strains were kindly provided by J. H. Miller and have been described (24). GM-1-(F *lacI^{Q}L8, pro/ara, Δlac-pro, thi*) was used as repair-proficient *E. coli* K-12 strain, in which the Pt-induced mutations were introduced. The RecA strain was GM-1, carrying the *recA56* (28) mutation. The excision-repair-deficient strain was GM-1, *ΔchlA-uvrB-bioFCD*. The deletion in this strain originates from strain C261 described by Cleary *et al.* (29) and was transferred from that strain into GM-1 by P1 transduction.

RESULTS

Survival After Treatment with *cis*- and *trans*-$Pt(NH_3)_2Cl_2$. The survival curves for the strains with various repair capacities after treatment with *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ are given in Fig. 1. The bacterial cells were much more sensitive to the *cis* compound than to the *trans* compound. Furthermore, there was a striking difference in sensitivity between the repair-deficient UvrB and RecA cells and the repair-proficient cells toward *cis*-$Pt(NH_3)_2Cl_2$. These results will be discussed in relation to the specific action of *cis*-$Pt(NH_3)_2Cl_2$ on tumor cells.

LacI Forward Mutagenesis. Mutagenesis induced by *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ was examined using the phenyl galactoside selection method. The treated cells were directly plated on the phenyl galactoside plates. Additon of limiting amounts of glucose (up to 0.025%) to permit phenotypic expression of the induced mutations led to strong background growth but not to an increased number of mutant colonies on the plates. The absolute number of mutant colonies per plate was increased by treatment of repair-proficient cells with higher concentrations of *cis*-$Pt(NH_3)_2Cl_2$ (Table 1).

cis-$Pt(NH_3)_2Cl_2$ induced *lacI* mutations in the wild-type strain and in the RecA strain (Fig. 2) but not in the UvrB strain. At equitoxic doses, however, the *trans* compound did not induce these mutations. Subsequently, the occurrence of amber and ocher mutations among the induced *lacI* mutations was determined. This type of mutation results from base-pair substitutions. In the wild-type strain at 6% survival, 13% of a total of 5000 isolated mutants, derived from five independent cultures after treatment with *cis*-$Pt(NH_3)_2Cl_2$ at 50 μg/ml, were found to be either amber or ocher mutants. In the RecA strain, at a survival of 0.1% (1 μg/ml), when mutation induction is sufficiently high, no amber or ocher mutants were detected among 2000 isolated mutants. Apparently, the induction of base-pair substitutions is dependent on both the excision-repair system (blocked by a *uvrB* mutation) and a repair system that is dependent on RecA activity.

To determine at which sites the base-pair substitutions in the *lacI* gene of repair-proficient *E. coli* cells occur, 450 amber and 200 ocher mutants were further analyzed. By means of the LacI test system, the exact positions of the amber and ocher muta-

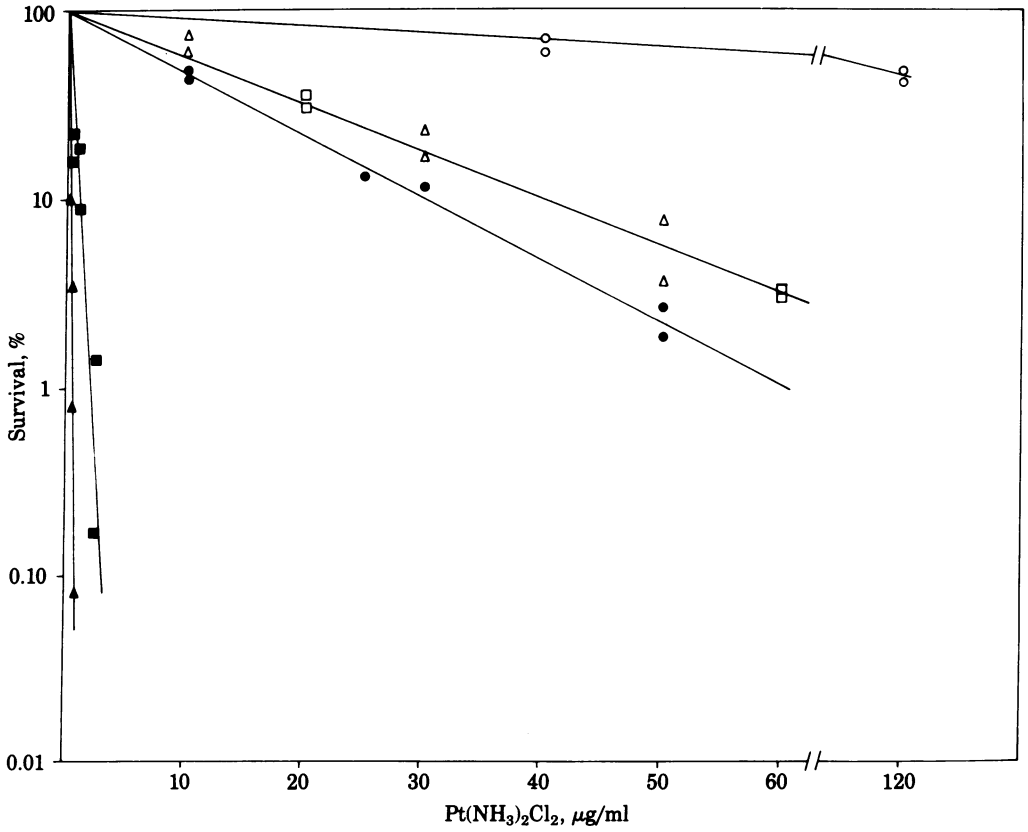

FIG. 1. Survival curves for different *E. coli* cells upon treatment with *cis*-$Pt(NH_3)_2Cl_2$ (●, repair-proficient; ▲, RecA; ■, UvrB) and *trans*-$Pt(NH_3)_2Cl_2$ (○, repair-proficient; △, RecA; □, UvrB). The survival was determined by plating the cells on LC agar. As shown in Table 1, survivals on LC agar and phenyl galactoside agar supplemented with 1% lactose were not different.

Table 1. Mutation induction and survival after treatment of repair-proficient cells with *cis*-$Pt(NH_3)_2Cl_2$

cis-$Pt(NH_3)_2Cl_2$, μg/ml	Survival, %*		*lacI* mutants, no./plate†		Induced mutation frequency, no./10^7 survivors
	LC	Phenyl galactoside + 1% lactose	Induced	Spontaneous	
0	100	100	84	84	—
5	66.0	69.5	238	109	35
25	12.7	13.2	354	56	213
50	1.7	1.8	843	42	2110

The survival on LC plates was only slightly different from that on phenyl galactoside/lactose plates. Therefore, the induced mutation frequencies in this and all other experiments are calculated from survival data obtained on LC plates.

* 100% = 1.1×10^9 cells per ml.

† Mean of experiments performed in triplicate; 100 μl of the undiluted, treated, or not-treated culture was plated on phenyl galactoside medium.

tions in the known base sequence of the *lacI* gene can be established. The resulting spectra are given in Fig. 3 for the amber mutations and in Fig. 4 for the ocher mutations. In the amber spectrum A2 and A17 are two predominant hot spots, followed by the less frequently occurring mutations in A27 and A6; in the ocher spectrum, O17, O25, and O32 are clearly hot spots. All the hot spots and also several less-frequently induced amber and ocher sites have a common feature—i.e., they all arise from GC → AT or GC → TA substitutions at sites where, except at A6, the guanine is part of a GAG or GCG sequence (Table 2). The only site at which mutagenesis occurs at a high level, but where the guanine is not in a GAG or GCG sequence, is A6. However, A6 is also known to be a spontaneous hot spot due to deamination of a 5-methylcytosine residue (30). Of the analyzed amber and ocher mutants, 70% originate from mutations in a GAG or GCG sequence.

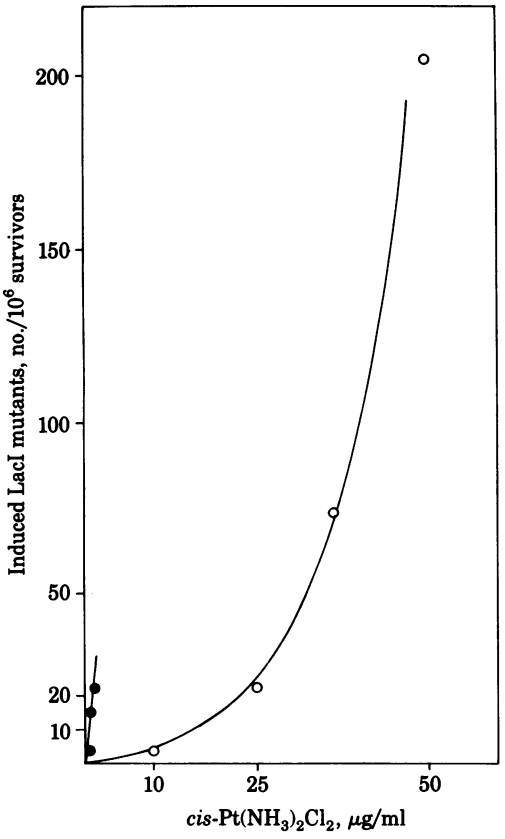

FIG. 2. Induction of LacI mutants upon treatment with *cis*-$Pt(NH_3)_2Cl_2$. ○, *E. coli* repair-proficient cells; ●, *E. coli* RecA cells.

DISCUSSION

Base-pair substitution mutations induced by *cis*-$Pt(NH_3)_2Cl_2$ that are scored as amber and ocher mutants occur preferentially at sites containing a GAG or GCG sequence in the DNA. Ten GAG and four GCG sites that mutable to amber or ocher codons are available in the *lacI* gene. At seven of these GAG sites and at three of these GCG sites, Pt-induced mutations are generated. The various sites containing these sequences, however, show a great variation in induced mutation frequencies (Table 1). The origin for this variation is not known, but it might be due to differences in the sequences of the neighboring bases, as was found in other mutation systems (31), or to the local conformation of the DNA.

Both *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ are known to form monoadducts with guanines in the DNA with nearly equal efficiency (7). Because *trans*-Pt compounds are hardly mutagenic it has been suggested that mutagenesis induced by the *cis* compounds occurs preferentially as a result of bifunctional finding (3). If mutagenesis is the result of binding of the *cis*-Pt compounds to either of two adjacent guanines in the same strand or two guanines in opposite strands, then potential amber and ocher sites with a guanine in a GG or GC sequence should be preferentially induced. Of the 10 sites in the *lacI* gene with appropriate GG sequences, only 2 (A17 and A6) are hot spots; but at hot spot A17, the substituted guanine is also part of a GAG sequence. So, A6 is the only frequently induced mutant in which the substituted guanine is not located in a GAG or GCG sequence.

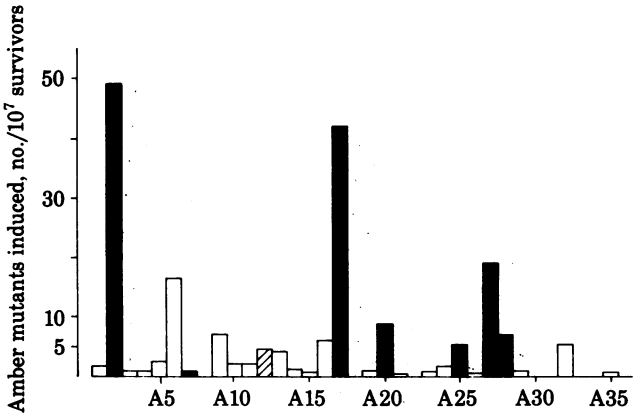

FIG. 3. Spectrum of amber mutants induced upon treatment of *E. coli* repair-proficient cells with *cis*-$Pt(NH_3)_2Cl_2$ at 50 μg/ml. Horizontally, the 36 possible amber sites in the *lacI* gene are indicated. The solid or hatched bars indicate that the substituted base pair is part of a GAG- or GCG-base sequence, respectively. Open bars represent all other sites mutable to amber codons.

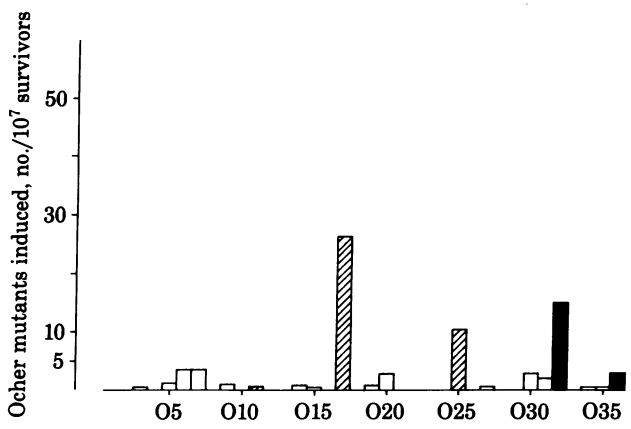

FIG. 4. Spectrum of ocher mutants induced upon treatment of *E. coli* repair-proficient cells with *cis*-$Pt(NH_3)_2Cl_2$ at 50 μg/ml. Horizontally, the 36 possible ocher sites in the *lacI* gene are indicated. The solid or hatched bars indicate that the substituted base pair is part of a GAG- or GCG-base sequence, respectively. Open bars represent all other sites mutable to ocher codons.

However, this A6 site is known to be a hot spot for spontaneous mutagenesis, due to the deamination of 5-methylcytosine (30), which partly explains the induction of the A6 mutants. Possibly, this deamination is enhanced by the binding of *cis*-$Pt(NH_3)_2Cl_2$. Concerning the interstrand crosslinks, only in 5 of the 22 appropriate GC sequences were mutations induced with a frequency above the background level. In all these cases, the guanines are also part of either a GAG or a GCG sequence (see Table 2: A20, A27, A28, O17, O25). Therefore, it seems unlikely that base-pair substitution mutagenesis occurs as a result of either intrastrand crosslinks on two adjacent guanines or interstrand crosslinks on two guanines in opposite strands.

The GAG and GCG sequences in the hot spots caused by *cis*-$Pt(NH_3)_2Cl_2$ strongly suggest that intrastrand crosslinks between two guanines separated by a third base are responsible for the base-pair substitution mutagenesis. As shown by molecular models, such a lesion is only possible if the base between the guanines becomes unstacked, resulting in the formation of a microloop in the DNA. Our results do not provide conclusive evidence as to whether or not the middle base can also be a guanine or a thymine, because only one GTG sequence (O9), which was slightly induced, and only one GGG sequence (O26), which was not induced, are available in the *lacI* gene at a site where an amber or ocher codon can be formed.

The proposed intrastrand crosslink between two guanines in a GAG or GCG sequence, which so far has not been reported, is in agreement with a number of earlier observations: (*i*) preference of Pt compounds to bind in GC-rich DNA regions (35); (*ii*) strong perturbation of the DNA structure upon bidentate Pt binding (8); (*iii*) decrease of the melting temperature (t_m) of DNA after binding of *cis*-Pt compounds (8, 36); (*iv*) shortening of DNA molecules up to 50%, depending upon the concentration of the Pt compound (8, 37); (*v*) selective inhibition of the cutting at a particular site in Pt-treated plasmid DNA by a restriction enzyme as found by Cohen *et al.* (38) [these authors ascribed this effect to the presence of a $(dG)_4 \cdot (dC)_4$ cluster near the restriction site; however, in the light of the present results, it is of interest, that this part of the DNA contains also a unique GAG sequence].

Although the proposed formation of crosslinks on GAG and GCG sites contributes substantially to the total amount of mutagenesis in wild-type *E. coli* by the induction of base-pair substitutions, other types of lesions formed by the action of *cis*-Pt compounds cannot be excluded. If these occur, they do not contribute to the major type of mutagenesis and the corre-

Table 2. Neighboring base-sequences of potential amber and ocher sites in the *lacI* gene and the frequencies of *cis*-$Pt(NH_3)_2Cl_2$-induced mutations at these sites

Site	Sequence	Mutations, no. induced/ 10^7 survivors
A2	GCA*G*AGTAT CGT*C*TCATA	49.3
A6	AAC*C**AGGCC TTG*G*TCCGG	16.5
A7	GCG*G*AGCTG CGC*C*TCGAC	0.8
A12	CCGTCGCAA GGCA*G*CGTT	4.3
A17	GTGGAGCAT CAC*C*TCGTA	42.7
A20	GTCTCGGCG CAGA*G*CCGC	8.7
A25	AATGAGGGC TTA*C*TCCCG	5.2
A27	ACCGAGTCC TGG*C*TCAGG	18.8
A28	ATCTCGGGT TAGA*G*CCCA	7.0
A33	TCTCAGGGC AGA*G*TCCCG	0.0
O9	GCA*C*AACAA CGT*G*TTGTT	1.8
O11	TCG*C*AAATT AGC*G*TTTAA	0.4
O17	GCG*C*AACGC CGC*G*TTGCG	26.2
O25	GCG*C*AACGG CGC*G*TTGCC	10.2
O26	CGG*G*AACGG GCC*C*TTGCC	0.0
O32	AGCTCATGT TCGA*G*TACA	19.6
O36	GTCTCACTG CAGA*G*TGAC	2.7

The table includes the potential amber and ocher sites at which the base pair that has to be replaced to form a nonsense codon is part of a GXG sequence (X = A, T, C, or G); it also includes A6 (see text). The base sequences are as reported by Farabaugh (32), except for O25, at which the sequence is in accordance with the amino acid sequence described by Beyreuther *et al.* (33, 34). To obtain the nonsense codons, the italic base pairs have to be replaced. The observed mutation frequencies were determined after treatment of repair-proficient cells with *cis*-$Pt(NH_3)_2Cl_2$ at 50 μg/ml in buffer for 2 hr at 37°C.
* 5-Methylcytosine.

sponding Pt adducts may be eliminated by error-free repair or may directly lead to lethality.

cis-$Pt(NH_3)_2Cl_2$ induces mutagenesis not only in wild-type cells but also in RecA cells. However, the mutants induced in RecA cells are not the result of base-pair substitutions because the frequency of amber and ocher mutations after treatment with the Pt compound does not exceed the spontaneous frequency.

In UvrB cells no mutagenesis was found. Apparently, base-pair substitution mutagenesis in repair-proficient cells is dependent on both UvrB and RecA. This implies that the first step is most likely the recognition of the distortion of the DNA helix by the UV-endonuclease, resulting in incision. For base-pair substitutions in repair-proficient cells, the incision is probably followed by a RecA-mediated error-prone process. Two repair pathways, both dependent on UvrB and RecA, have been reported in the literature. (*i*) Incision, followed by recombination, as a mechanism for repair of interstrand crosslinks (39, 40), which according to our results does not cause base-pair substitutions. (*ii*) Long-patch DNA repair (41), a pathway that plays only a minor role in the removal of pyrimidine dimers in the so-called excision-repair process but might be a more important pathway for the repair of other chemically induced lesions (41). Long-patch DNA repair is considered to be a SOS-type of repair (42, 43). *cis*-$Pt(NH_3)_2Cl_2$ is known to induce several SOS responses (44), such as prophage induction (45), filamentous growth in bacteria (46), and selective inhibition of DNA replication (4, 5). Therefore, long-patch DNA repair could be the explanation for the occurrence of base-pair substitution mutants, induced by *cis*-$Pt(NH_3)_2Cl_2$.

To explain the selectivity of *cis*-Pt compounds toward tumor cells, it has been proposed that rapidly dividing cancer cells are more sensitive than normal cells to DNA synthesis-inhibiting agents (47). However, this does not explain why only certain tumors give a positive response upon treatment with the *cis*-Pt compounds (3). Fraval and Roberts (48) demonstrated that Chinese hamster cells are more sensitive to DNA-bound Pt in the stationary (G_1) phase than in the growing (mid-S) phase and ascribed this feature to a lower efficiency of the excision repair in the stationary phase. As shown in this investigation, repair-deficient *E. coli* cells are extremely sensitive *cis*-$Pt(NH_3)_2Cl_2$. The present results further support the suggestion (49, 50) that the specific action of *cis*-Pt compounds toward some types of tumor cells is due to a repair deficiency in these cells.

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