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

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ABSTRACT Cell killing and mutation induction by cis- and trans-Pt(NH3)2Cl2 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(NH3)2Cl2 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(NH3)2Cl2 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 GCC 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(NH3)2Cl2 shows antitumor activity against sarcoma 180 and leukemia L1210, whereas trans-Pt(NH3)2Cl2 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(NH3)2Cl2 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(NH3)2Cl2 binds to bases in DNA, and the order of binding affinity has been shown to be guanine>B>adenine>C>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(NH3)2Cl2 exerts its antitumor activity because, at nontoxic doses, more of the inactive trans compound is bound to DNA (7). Therefore, specific bifunctional binding of cis-Pt(NH3)2Cl2 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), intersstrand crosslinking between the N-7 positions of guanines in opposite strands (3, 7, 9), and intrasstrand 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(NH3)2Cl2-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 conjuga- tion of the F' epispores 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 ochre (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(NH3)2Cl2 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
RESULTS

Survival After Treatment with cis- and trans-Pt(NH$_3$)$_2$Cl$_2$.

The survival curves for the strains with various repair capacities after treatment with cis- and trans-Pt(NH$_3$)$_2$Cl$_2$ 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$_3$)$_2$Cl$_2$. These results will be discussed in relation to the specific action of cis-Pt(NH$_3$)$_2$Cl$_2$ on tumor cells.

LacI Forward Mutagenesis. Mutagenesis induced by cis- and trans-Pt(NH$_3$)$_2$Cl$_2$ 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$_3$)$_2$Cl$_2$ (Table 1).

cis-Pt(NH$_3$)$_2$Cl$_2$ 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 ochre 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$_3$)$_2$Cl$_2$ at 50 μg/ml, were found to be either amber or ochre mutants. In the RecA strain, at a survival of 0.1% (1 μg/ml), when mutation induction is sufficiently high, no amber or ochre 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 ochre mutants were further analyzed. By means of the LacI test system, the exact positions of the amber and ochre muta-
mutations 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 ochre 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 ochre spectrum, O17, O25, and O32 are clearly hot spots. All the hot spots and also several less-frequently induced amber and ochre 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 ochre mutants, 70% originate from mutations in a GAG or GCG sequence.

**DISCUSSION**

Base-pair substitution mutations induced by cis-Pt(NH$_3$)$_2$Cl$_2$ that are scored as amber and ochre 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 ochre 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$_3$)$_2$Cl$_2$ 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 ochre 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.

**Table 1. Mutation induction and survival after treatment of repair-proficient cells with cis-Pt(NH$_3$)$_2$Cl$_2$**

<table>
<thead>
<tr>
<th>cis-Pt(NH$_3$)$_2$Cl$_2$, μg/ml</th>
<th>Phenyl galactoside + 1% lactose</th>
<th>lacI mutants, no./plate$^*$</th>
<th>Induced mutation frequency, no./10$^7$ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>LC</td>
<td>84</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>235</td>
<td>35</td>
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<tr>
<td>25</td>
<td>12.7</td>
<td>354</td>
<td>213</td>
</tr>
<tr>
<td>50</td>
<td>1.7</td>
<td>843</td>
<td>2110</td>
</tr>
</tbody>
</table>

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$^6$ 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.

**Fig. 2.** Induction of LacI mutants upon treatment with cis-Pt(NH$_3$)$_2$Cl$_2$. c, E. coli repair-proficient cells; o, E. coli RecA cells.

**Fig. 3.** Spectrum of amber mutants induced upon treatment of E. coli repair-proficient cells with cis-Pt(NH$_3$)$_2$Cl$_2$ 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.
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(NH₃)₂Cl₂. 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 base 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(NH₃)₂Cl₂ 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 GTC sequence (O9), which was slightly induced, and only one GCG sequence (O26), which was not induced, are available in the lacI gene at a site where an amber or other 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 (Tₘ) 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(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-
sponding Pt adducts may be eliminated by error-free repair or may directly lead to lethality.

cis-Pt(NH₃)₂Cl₂ 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 ochre 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(NH₃)₂Cl₂ 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(NH₃)₂Cl₂.

To explain the selectivity of cis-Pt compounds toward tumor cells, it has been proposed that rapidly dividing cancer cells are more sensitive to 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 (G₁) 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 to cis-Pt(NH₃)₂Cl₂. 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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