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Enhanced DNA Repair as a Mechanism of Resistance to *cis*-Diamminedichloroplatinum(II)†

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ABSTRACT: Murine leukemia L1210 cells, either sensitive or resistant to the toxic action of the cancer chemotherapeutic agent *cis*-diamminedichloroplatinum(II), have been studied for potential differences in the formation and repair of drug-induced DNA damage. The sensitivity for these experiments was obtained by using the radiolabeled analogue [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II). The resistant cells demonstrated a 40% reduction in drug accumulation but a qualitatively similar profile of DNA-bound adducts. These adducts resembled those previously characterized in pure DNA and represented intrastrand cross-links at GG, AG, and GNG (N is any nucleotide) sequences in DNA. Repair of these cross-links occurred in a biphasic manner: rapid for the first 6 h and then much slower. The resistant cells removed up to 4 times as many adducts during the rapid phase of repair. The extent of this repair did not directly correlate with the degree of resistance in that cells with 100-fold resistance were only slightly more effective at repair than cells with 20-fold resistance. Therefore, although enhanced DNA repair is thought to contribute markedly to drug resistance, other mechanisms for tolerance of DNA damage may also occur in these cells.

Development of resistance to cancer chemotherapeutic agents is a major limitation to the clinical use of these drugs. Many experimental cell systems have been developed to investigate the potential mechanisms of resistance to *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] [reviewed in Eastman and Richon (1986)]. Various patterns of cross-resistance have been reported. Cells may be either specifically resistant to *cis*-DDP or also cross-resistant to other alkylating agents or even cadmium. In the latter cases, alterations in glutathione or metallothionein levels have been implicated in the resistance mechanism.

In the past year, a number of reports have suggested that reduced accumulation of *cis*-DDP may contribute, albeit only partially, to the mechanism of resistance (Kraker et al., 1986; Hromas et al., 1986; Andrews et al., 1986). This laboratory demonstrated that murine leukemia L1210 cells preferentially resistant to *cis*-DDP (L1210/DDP) have a slightly reduced accumulation of drug but the major contribution to resistance occurs after DNA is platinated (Richon et al., 1987).

DNA is believed to be the critical biological target (Roberts & Thomson, 1979). Damage in the form of specific DNA adducts has been characterized (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985). The major adducts are DNA intrastrand cross-links, but the DNA interstrand cross-links that represent less than 1% of the total platination have more often been implicated as the most cytotoxic lesions [reviewed in Eastman (1987a)]. In bacteria, sensitivity to *cis*-DDP can arise by a deficiency in repair of the damaged DNA (Drobnik et al., 1973; Beck & Brubaker, 1973). Additionally, Sancar and Rupp (1983) showed that the *Escherichia coli* uvrABC excinuclease complex can excise *cis*-DDP adducts from DNA in vitro. A deficiency in cellular repair processes in mammalian cells also contributes to sensitivity to DNA-damaging agents (Fraval et al., 1978; Meyn et al., 1982). The corollary that sensitivity to DNA damage is associated with deficient DNA repair does not hold for every cell line (Rawlings & Roberts, 1986). Thus, DNA repair processes are important determinants of the cytotoxic effects of DNA-damaging agents. However, neither in bacteria nor in mammalian cells has an increase in DNA repair capability been reported as a mechanism of resistance to genotoxic agents.

In the present study, we have investigated potential differences in DNA repair in L1210/0 (sensitive) and L1210/DDP cell lines. These experiments involved analyzing the formation and repair of specific DNA intrastrand cross-links. The sensitivity for these experiments was obtained by using [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II) ([^{3}H]-*cis*-DEP), an analogue of *cis*-DDP that produces adducts at identical sites in DNA [reviewed in Eastman (1987a)] and

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *cis*-DEP, *cis*-dichloro(ethylenediamine)platinum(II); HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Table I: Properties of Cell Lines and Levels of Platination

cell line	IC_{50}[a] (μg/mL)	x-fold resistance	intracellular drug concn[b] (pmol/10^7 cells)	DNA platination[c] (nmol of Pt/g of DNA)
L1210/0	0.12		3.1	0.33
L1210/DDP_2	2.7	23	1.9	0.27
L1210/DDP_5	5.8	48	1.8	0.23
L1210/DDP_{10}	13.2	110	1.7	0.21

[a] This value is after a 3-day incubation in *cis*-DDP [from Richon et al. (1987)]. [b] The amount of dissociation of drug in the cells is unknown. These values have not been corrected for this. [c] These values are corrected for 10% incorporation of tritium into normal DNA.

to which L1210/DDP cells are equally resistant (Eastman & Bresnick, 1981).

Materials and Methods

Unlabeled *cis*-DEP was purchased from Alfa Ventron, Danvers, MA. The preparation of [^{3}H]-*cis*-DEP was as previously detailed (Eastman, 1983). Thiourea was stored as a 1 M solution over AG501-X8 (Bio-Rad, Richmond, CA). All enzymes were purchased from Sigma Chemical Co., St. Louis, MO.

Sensitive murine leukemia L1210/0 cells and the *cis*-DDP-resistant sublines L1210/DDP_2, L1210/DDP_5, and L1210/DDP_{10} have been previously described (Richon et al., 1987). These cell lines are maintained in 2, 5, and 10 μg/mL cis-DDP, respectively, and are approximately 20-, 50-, and 100-fold resistant compared to L1210/0 (Table I).

To assess the extent of DNA platination and subsequent repair, approximately 5×10^6 cells were incubated in 40 mL of McCoy's 5a (modified) medium with 5 nCi of [^{14}C]thymidine. After 48–72 h, the cells were aliquoted for drug treatment. In each experiment, 10^7 cells in 10 mL of culture medium were incubated at 37 °C for 6 h with 0.05 μg/mL [^{3}H]-*cis*-DEP (2.5 μCi/mL). The cells were washed and harvested or incubated for up to 24 h in drug-free medium before harvesting. DNA was purified from the cells by a modification of Eastman et al. (1982). Cells were sedimented at 900*g* for 5 min and the pellets dispersed by sonication (2 × 10-s periods, 70 W, Heat Systems Ultrasonic Inc.) in 5 mL of 2 M NaCl/5 M urea/200 mM sodium phosphate, pH 6.8. A 100-μL aliquot was scintillation counted to assess total radioactivity associated with the cell pellet. The remainder was applied to a 3-mL hydroxyapatite column that had been fined and equilibrated with the same buffer. Elution with 10 mL of 300 mM sodium phosphate/2 M NaCl/5 M urea, pH 6.8, removed the majority of protein and RNA. DNA was eluted in 6 mL of 600 mM sodium phosphate/5 M urea, pH 6.8 (no NaCl). The elution flow was maintained by gravity and took about 1 h to complete. The DNA fraction was dialyzed against three changes of H_2O, lyophilized, redissolved in 1 mL of 10 mM Tris/1 mM EDTA, pH 7.4, and digested at 37 °C for 1 h with 100 μg/mL ribonuclease A followed by 1 h with 100 μg/mL proteinase K. This was followed by two extractions with chloroform/isoamyl alcohol (5:1 v/v), and the DNA was precipitated with 2 volumes of ethanol/2% potassium acetate. The pellet was washed with 70% ethanol/2% potassium acetate and redissolved in 0.6 N perchloric acid by heating at 80 °C for 30 min. The absorbance at 260 nm and radioactivity were determined.

For the analysis of specific DNA-bound adducts, the cells were incubated with 10 μCi of [^{3}H]-*cis*-DEP/mL. After purification, the DNA pellet was redissolved in 200 μL of 50 mM sodium acetate/10 mM $MgCl_2$, pH 5.5, and digested to deoxyribonucleosides, and the products were separated by HPLC as previously detailed (Eastman, 1986).

Results

Drug Accumulation and DNA Platination. We have previously demonstrated that the resistant cells used in these studies accumulate reduced levels of [^{3}H]-*cis*-DEP (Richon et al., 1987). The present studies have analyzed the effect of reduced accumulation on the levels of drug adducted to DNA. The various cell lines were labeled with [^{14}C]thymidine and then incubated with [^{3}H]-*cis*-DEP for 6 h. Aliquots of cells harvested at this time demonstrated that the L1210/DDP cells accumulated about 40% less tritium than the sensitive cells (Table I). Cells harvested after a further 24-h incubation in drug-free medium demonstrated that 70–80% of the tritium had disappeared from all the cell lines.

The DNA was purified from these cells and assayed for quantity of DNA (A_{260}) and radioactivity. The amount of tritium bound to DNA after the 6-h incubation demonstrated that all the resistant cell lines had a slight reduction in the amount of bound drug compared to L1210/0 cells (Table I). As discussed below, some of the tritium associated with DNA is not due to adduction by drug but is due to metabolism of the drug and incorporation of tritium into normal DNA. However, at 6 h, this represents only 10–15% of the total tritium in DNA; the remainder is identifiable as specific adducts.

Both drug accumulation and DNA platination were linear up to 30 μg/mL. The approximate concentrations of *cis*-DEP that, following a 6-h incubation, inhibited growth by 50% (IC_{50}) as measured over 3 days were 0.5 μg/mL for L1210/0 cells and 25 μg/mL for L1210/DDP_{10} cells. Correcting for the proportion of tritium in DNA that was not attributed to adducts, it was calculated that at the IC_{50} value for L1210/0 cells there were 1.7×10^4 molecules of *cis*-DEP adducted to the DNA of each cell, i.e., 1 Pt/10^6 nucleotides. This value is in the range previously reported for a variety of cell lines and *cis*-DDP analogues (Johnson et al., 1986; Roberts et al., 1986). At the IC_{50} value for L1210/DDP_{10} cells, there were 8.6×10^5 molecules adducted to the DNA, i.e., 1 Pt/2×10^4 nucleotides. These values confirmed the contention that the resistant cells were able to survive in the presence of much higher levels of adducts in their DNA.

Monofunctional DNA Damage. Experiments were designed to test the possibility that resistance was attributable to alterations in monofunctional adducts. Thiourea treatment has previously been used to rescue cells from toxicity induced by *cis*-DDP (Zwelling et al., 1979). In preliminary experiments, cells were incubated for 2 h with *cis*-DDP followed by incubation with various concentrations of thiourea for up to 1 h. Growth inhibition was then measured over the following 3 days. It was found that a 20-min incubation in 100 mM thiourea produced the maximum rescue from the *cis*-DDP-induced inhibition of growth. Comparison of L1210/0 and L1210/DDP_{10} cells under these conditions showed that a significant rescue of the cells was achieved but that the extent of rescue was similar in both cell lines (Figure 1A,B). The rate of rearrangement of these monofunctional adducts to more toxic, bifunctional adducts was investigated by incubating the cells for up to 8 h between treatment with *cis*-DDP and thiourea (Figure 1C). After 5 h, thiourea no longer could rescue the cells from the toxicity of *cis*-DDP in either sensitive or resistant cells. There was no indication that monofunctional adducts might be preferentially repaired in the resistant cells; in fact, the monofunctional adducts appeared slightly more persistent in the resistant cells, although this is probably not a significant difference.

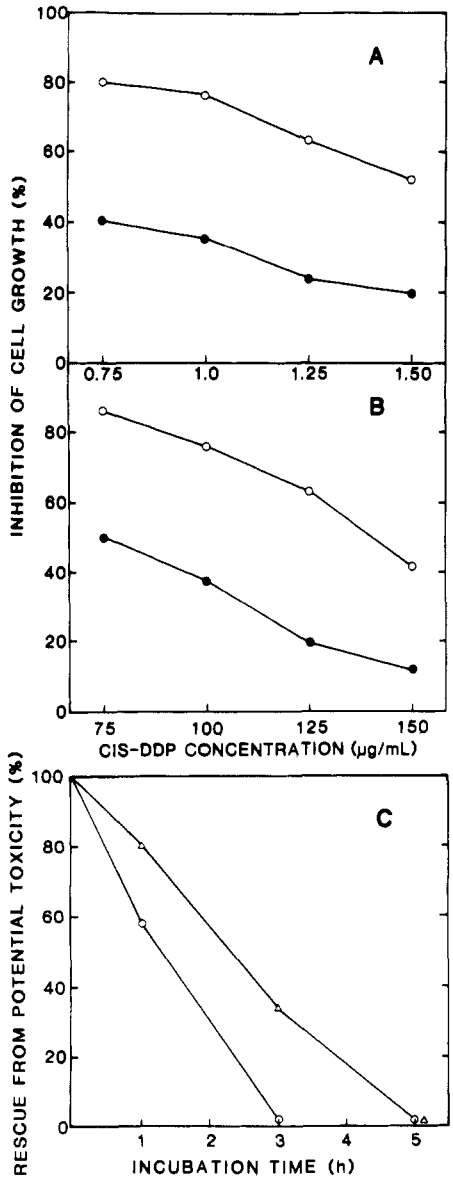

FIGURE 1: Rearrangement of monofunctional DNA-bound adducts to more toxic lesions as assessed by the ability of thiourea to rescue cells from *cis*-DDP-induced inhibition of growth. (A) L1210/0 and (B) L1210/DDP_{10} cells were incubated with *cis*-DDP for 2 h and then with (open symbols) or without (closed symbols) 100 mM thiourea for 20 min. Growth of cells was assessed over the subsequent 3 days. (C) The rate of rearrangement was assessed by the loss of the ability of thiourea to rescue L1210/0 (○) and L1210/DDP_{10} (△) cells from 1 and 100 μg/mL *cis*-DDP, respectively. Incubation time refers to the period between incubation with *cis*-DDP and thiourea.

Characterization of DNA Adducts in L1210 Cells. The profile of DNA-bound adducts was investigated by incubating cells with 0.2 μg/mL [^{3}H]-*cis*-DEP (10 μCi/mL) for 6 h. The DNA was purified and digested to deoxyribonucleosides, and the products were separated by HPLC. The elution buffer contained either 0.2 or 0.02 M ammonium acetate. In the low-salt condition, charged adducts elute later, which facilitates both their resolution and characterization (Eastman, 1986). The HPLC profile of the adducts produced in L1210/0 cells is shown in Figure 2A,B. Seven peaks have been numbered for reference. Peaks 4, 6, and 7 chromatograph identically with the three adducts produced in pure DNA [compare to Figure 1 of Eastman (1986)]. These represent respectively platination at GG and AG and a cross-link between two deoxyguanosines. This latter adduct is derived mainly from platination at GNG (N is any nucleotide). Peak 1 cochromatographed with a cross-link between deoxyguanosine and gluathione (Eastman, 1987b). The identity of peaks 2, 3, and 5 is described below. The profile of adducts obtained from L1210/DDP_{10} cells incubated with [^{3}H]-*cis*-DEP for 6 h showed only slight quantitative differences (Figure 2C,D).

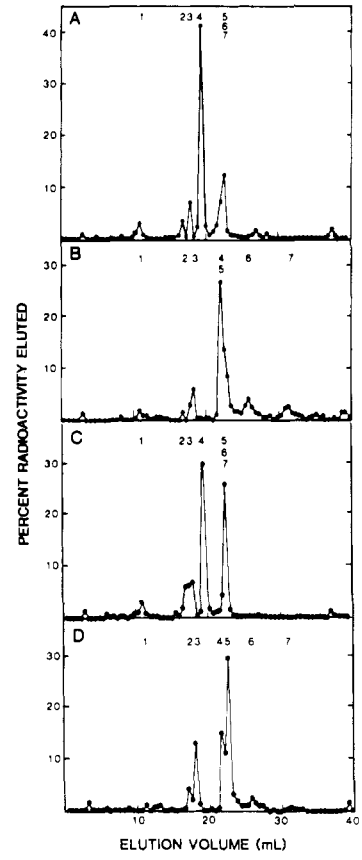

FIGURE 2: HPLC separation of DNA-bound adducts produced in L1210/0 (A and B) and L1210/DDP_{10} (C and D) cells incubated for 6 h with [^{3}H]-*cis*-DEP. The HPLC elution buffer was 0.2 M (A and C) or 0.02 M (B and D) ammonium acetate. The level of DNA modification for each cell line is presented in Table I. The identity of the numbered peaks is described in the text.

After the L1210/0 cells had recovered for 24 h, the radioactive profile was very similar to that at 6 h except for a slight increase in peaks 3 and 5 (Figure 3A,B). The profile derived from the L1210/DDP cells was markedly different. The adduct at GG had almost completely disappeared, while adducts at AG and GNG were undetectable. Peak 5 was the major peak (Figure 3C,D).

These new radiolabeled peaks were characterized as follows. They were purified by HPLC, incubated with 1 M thiourea to remove drug, and reanalyzed by HPLC. No alteration occurred in the chromatographic mobility of the radioactivity, suggesting that no platinum was present. On both reverse-phase and cationic exchange columns, the radioactivity of peaks 3 and 5 cochromatographed with unmodified thymidine and deoxyadenosine. These peaks, therefore, result from dissociation of the drug, metabolism of [^{3}H]ethylenediamine, and incorporation into DNA as the normal deoxyribonucleosides. Peak 2 cochromatographed with deoxyguanosine by reverse-phase HPLC. No further confirmation of this identity was attempted.

It was evident that the disappearance of peaks 4, 6, and 7 in these studies represnted a real enhancement in a DNA-repair process. To accurately quantify this process, we have analyzed specific ^{3}H adducts from experiments in which cells were prelabeled with [^{14}C]thymidine. This obviated the

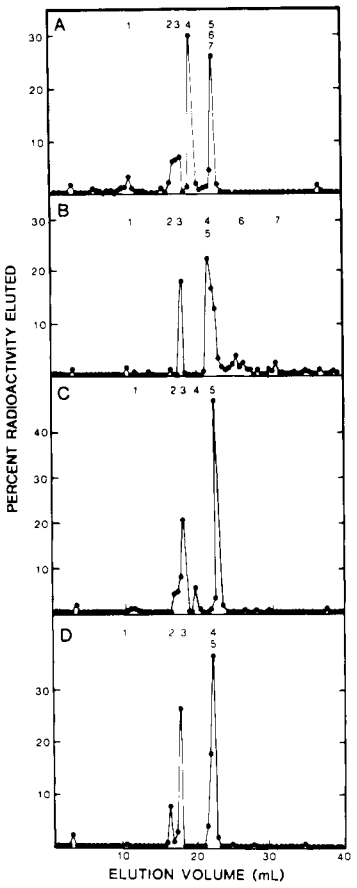

FIGURE 3: HPLC separation of DNA-bound adducts produced in L1210/0 (A and B) and L1210/DDP$_{10}$ (C and D) cells incubated with [^{3}H]-*cis*-DEP for 6 h and incubated for a further 24 h in drug-free medium. The HPLC elution buffer was 0.2 M (A and C) or 0.02 M (B and D) ammonium acetate.

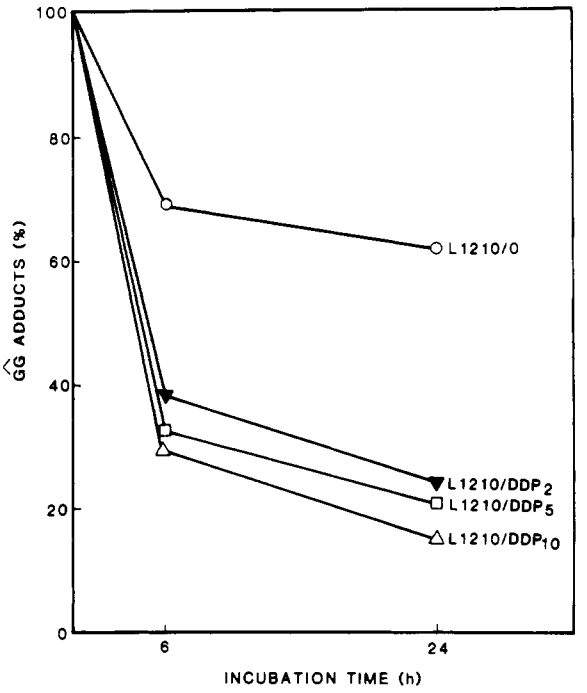

FIGURE 4: Repair of DNA adducts at GG sequences in L1210/0, L1210/DDP$_2$, L1210/DDP$_5$, and L1210/DDP$_{10}$ cells following a 6-h incubation with [^{3}H]-*cis*-DEP (100% platination). Values were obtained by purifying DNA, separating adducts by HPLC, and comparing the [^{14}C]thymidine peak with ^{3}H label associated with the adduct at GG.

contribution from both ^{3}H nucleosides and DNA replication. The rate of repair was defined as the change in the ratio of ^{3}H adduct to [^{14}C]thymidine, both quantified in a single HPLC separation. The repair of adducts at GG sequences was markedly enhanced in the resistant cells (Figure 4). L1210/0 cells repaired 30% of these adducts in 6 h, while L1210/DDP$_{10}$ cells repaired 70%. Repair during the following 18 h was considerably slower. The radioactivity associated with the other adducts was too low to accurately determine the rates of repair.

Discussion

The present studies have confirmed that L1210/DDP cells accumulated approximately 60% of the *cis*-DEP accumulated by the sensitive L1210 cells. This, in turn, led to a reduction in DNA platination. However, the resistant cells were able to tolerate 50 times the level of DNA adducts found in the sensitive cells. The level of DNA adducts in the sensitive cells was in the range generally considered to represent wild-type cells rather than hypersensitive cells. Accordingly, the L1210/DDP cells have truly acquired resistance. The frequency of monofunctional platination of DNA was indirectly measured by the ability of thiourea to rescue cells from the toxic events. No significant differences between the cell lines were detected in this parameter.

The platinated DNA was enzymatically digested, and the deoxyribonucleoside-bound adducts were separated by HPLC. The adduct profile was very similar to that previously reported for platination of pure DNA (Eastman, 1987a). The major adduct was at a GG sequence, with minor adducts at AG and GNG sequences. The adduct analysis was confounded, however, by the formation of unexpected radioactive peaks. These have been characterized as incorporation of radioactivity into normal deoxyribonucleosides, particularly deoxyadenosine. This is presumed to result from dissociation of the [^{3}H]-ethylenediamine from the drug during incubation, followed by metabolism and incorporation into DNA. The [^{3}H]-deoxyadenosine was readily separated by HPLC from ^{3}H adducts at GG, and this permitted an accurate assessment of the amount of DNA repair. The levels of DNA platination reported under Results have been corrected for the 10–15% incorporation of radiolabel as [^{3}H]deoxyadenosine into DNA during the 6-h incubation.

The most significant observation in these studies was the enhanced repair of adducts at GG seen in L1210/DDP cells. The adducts at AG and GNG were also repaired rapidly, but accurate calculation was limited by the low levels of radioactivity involved. Repair appears to occur in two distinct phases: an initial rapid phase followed by a slow or plateau phase. Hence, 24 h after platination, the L1210/DDP$_{10}$ cells have removed 4 times as many adducts from GG sequences as have the L1210/0 cells. Considering the additional contribution from reduced accumulation and lower initial DNA platination, the L1210/DDP$_{10}$ cells retain only 15% of these adducts as compared to the sensitive cells. Although the cells appeared to remove more adducts as resistance increased, there was no quantitative correlation. The majority of the enhanced repair occurred in cells 20-fold resistant to *cis*-DDP with only a 2-fold further increase in repair as they attained 100-fold resistance. This suggests that additional mechanisms of resistance may contribute to the high degree of resistance in L1210/DDP$_{10}$ cells.

A number of *cis*-DDP-resistant cell lines have been reported, and attempts have been made to assess the mechanisms of this resistance [reviewed in Eastman and Richon (1986)]. In most cases, some difference in drug accumulation has been reported. On occasion, alterations in glutathione or metallothionein

occur, but in each case it has been concluded that these mechanisms can only account for part of the resistance. During the course of these experiments, one other group reported a contribution of DNA repair to resistance, in that case by measuring unscheduled DNA synthesis in a human ovarian cell line (Behrens et al., 1987). A sequel to that report was the observation that aphidicolin, an inhibitor of DNA polymerase α, could overcome resistance, presumably by interfering with the polymerase step of DNA repair (Hamilton et al., 1987). The present studies provide further information in that the repair of specific DNA adducts has been followed. These results show enhanced repair of intrastrand cross-links, thereby suggesting their importance to toxicity. Comparison with our earlier work (Strandberg et al., 1982) suggests that interstrand cross-links may also be more rapidly repaired in L1210/DDP cells over a similar time course. The relative significance of each adduct to toxicity remains in contention. Intrastrand cross-links are efficient at inhibiting transcription of platinated plasmid DNA in L1210 cells (Sheibani & Eastman, 1987) as well as inhibiting DNA synthesis in platinated, defined DNA template (Eastman & Richon, 1986). The same is probably true of interstrand cross-links, but these assays are not specific or sensitive enough for such an assessment because interstrand cross-links form at less than 1% frequency of intrastrand cross-links (Eastman, 1987a). It will be necessary to isolate the enzymes involved in order to assess their substrate specificity and, hence, determine the lesions critical to toxicity. The same line of experimentation will be needed to determine whether other mechanisms contribute to the high degree of resistance to *cis*-DDP in the L1210/DDP cells. It is worth emphasizing that the enzyme(s) involved in this enhanced repair cannot be a normal DNA repair enzyme as the cells show no cross-resistance to almost all other DNA-damaging agents (Eastman & Bresnick, 1981). The present hypothesis is that the critical enzyme(s) result(s) from mutation of an otherwise normal DNA repair gene such that it recognizes the DNA damage with high efficiency.

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