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DNA Cross-Linking as an Indicator of Sensitivity and Resistance of Mouse L1210 Leukemia to *cis*-Diamminedichloroplatinum(II) and L-Phenylalanine Mustard

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ABSTRACT

The relationship between DNA cross-linking and cell killing by *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and L-phenylalanine mustard (L-PAM) was studied in L1210 cell culture lines and in mice bearing sensitive and resistant lines of L1210 leukemia. A line of L1210 mouse leukemia cells was developed which is resistant to *cis*-DDP *in vitro*. These cells, designated ZCR9, are cross-resistant to L-PAM. The effect of both drugs on the ZCR9 cells, compared to the parent L1210 K25 cells, was examined by DNA alkaline elution with and without the use of proteinase. The resistant line was similar to the normal line with regard to the kinetics of DNA cross-link formation and removal following treatment with *cis*-DDP or L-PAM. For both drugs, maximum cross-linking occurred after 6 hr; this is presumed to represent the time required for conversion of drug-DNA monoadducts to cross-links. In the resistant line, interstrand cross-linking by *cis*-DDP or L-PAM and DNA-protein cross-linking by *cis*-DDP were all reduced relative to the parent line. The interstrand cross-linking was reduced in approximately the same proportion as the cytotoxicity (in terms of dose modification factors). DNA-protein cross-linking by L-PAM, however, was similar in the two cell lines. The relationship between DNA cross-linking and cell killing by *cis*-DDP and L-PAM was also studied in mice bearing sensitive and resistant lines of L1210 leukemia. The cells were removed from untreated mice and tested *in vitro* for DNA cross-linking produced by the two drugs. Tumor sensitivity was assessed by comparing the survival of treated *versus* untreated mice which had been inoculated with the same cells used in cross-linking assays. A L1210 line which had been developed for resistance to *cis*-DDP exhibited marked reductions in both types of cross-linking by this drug when compared to its sensitive parent line. This line was not resistant to L-PAM and exhibited no significant depression in cross-linking by this drug. A second line, made resistant to L-PAM, showed marked reductions in L-PAM-induced cross-linking compared to its parent line. This line was cross-resistant to *cis*-DDP but showed only a modest reduction in *cis*-DDP-induced cross-linking. Thus, in three of the four cell-drug comparisons, DNA cross-linking and *in vivo* cell killing were well correlated. The reason for the deviation of the fourth case was investigated in preliminary studies, but no definitive answer was obtained. The results suggest that DNA cross-linking correlates with tumor sensitivity to bifunctional agents.

INTRODUCTION

cis-DDP,[2] like the bifunctional nitrogen mustards, is capable of undergoing bifunctional addition reactions with DNA, producing interstrand and intrastrand cross-links, as well as DNA-protein cross-links (6, 10, 11, 15–18). It is likely that one or more of these classes of bifunctional DNA lesions is responsible for the cytotoxicity and antitumor activity of these drugs.

Previous work in our laboratory has utilized the alkaline elution technique to study DNA interstrand and DNA-protein cross-linking in mammalian cells (5, 9). DNA cross-linking, especially of the interstrand type, correlates with cytotoxicity in several different cell types (3, 15–17). The kinetics of cross-link formation and removal following *cis*-DDP treatment was found to be generally similar to that following treatment with L-PAM (melphalan 15–17).[3] After treatment with either drug, cross-linking increases for a few hr and then slowly decreases. These 2 chemically different types of agents therefore lend themselves to comparative studies aimed at elucidating the roles of various types of cross-links in producing the biological effects.

In the current work, we have derived a resistant line of L1210 cells in culture and compared it with the parent line. The results suggested the possibility that tumor sensitivity to DNA cross-linking agents can be predicted by *in vitro* cross-linking measurements. This hypothesis was then tested in 2 sensitive/resistant pairs of L1210 tumor lines in mice.

MATERIALS AND METHODS

A cloned line (designated K25) of L1210 mouse leukemia cells was grown in RPMI Tissue Culture Medium 1630 (containing 0.03% glutamine, freshly added) plus 20% heated fetal calf serum as described previously (18). A *cis*-DDP-resistant line (designated ZCR9) was derived as follows. L1210 K25 cells were treated with 10^{-4} M methylnitrosourea for 1 hr. After recovery of exponential growth, the cells were treated with *cis*-DDP, and survivors were cloned in soft agar. A clone was selected, treated again with *cis*-DDP, and recloned in soft agar. An additional methylnitrosourea treatment and recovery was followed by 5 cycles of *cis*-DDP treatment and cloning. The colony survival levels following each treatment were 2.15×10^{-5} to 4.5×10^{-2}. Ampuls of the resulting resistant line (ZCR9) were stored in liquid nitrogen. The cells were grown for

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[2] The abbreviations used are: *cis*-DDP, *cis*-diamminedichloroplatinum(II); L-PAM, L-phenylalanine mustard; RPMI, Roswell Park Memorial Institute.

[3] W. E. Ross, L. A. Zwelling, and K. W. Kohn, unpublished observations.

no more than 6 weeks before going back to a fresh ampul. During this time, resistance was maintained.

Cell Treatments

cis-DDP and L-PAM were obtained through the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. *cis*-DDP (0.2 mM) was dissolved in RPMI Tissue Culture Medium 1630 plus 1% fetal calf serum and used within 1 hr. L-PAM (10 mM) was dissolved in 0.1 N HCl and stored frozen. Prior to drug treatment, exponentially growing cells were centrifuged and suspended in fresh RPMI Tissue Culture Medium 1630 containing 1% fetal calf serum. After 1 hr of drug exposure, the cells were centrifuged, washed, and resuspended in fresh medium containing 20% fetal calf serum. These procedures were carried out in a constant-temperature room at 37°.

Colony Assays

Colony-forming assays were carried out in soft agar by the method of Chu and Fisher (2). Cloning efficiencies of untreated cells were: K25, 91.6 ± 7.3% (S.D.); ZCR9, 75.4 ± 15.2%. The data were first plotted as log (survival fraction) against log (drug concentration) (Chart 1). In order to obtain the dose ratio that would make the curves for the 2 cell lines superimpose, the data points for each cell line were plotted on a separate sheet of semilogarithmic graph paper. Log (survival) was plotted on the linear scale, and drug concentration was plotted on the log scale. The 2 graph sheets were held up to the light, superimposed, and then displaced along the logarithmic concentration scale until the data points appeared most closely to fall on a common curve. The dose modification factor required to superimpose the data was then read off from the logarithmic scale of the graph paper as if using a slide rule. The data were then plotted in the usual manner for survival curves, *i.e.*, log (survival fraction) against drug concentration on a linear scale, but with concentration scales adjusted according to the dose modification factor (Chart 2).

Cell Proliferation Assays

Following drug treatment, washed cells were suspended in fresh medium at 2 to 3 × 10^5/ml, and the cell concentration was determined by means of an electronic cell counter. After 24 hr, the cells were counted again. Control cells grew exponentially during this period with 11- to 13-hr doubling times. The proliferation rate of treated relative to control cells was calculated by

$$\text{Fraction of control growth} = \frac{\left(\log \frac{N}{N_0}\right)_{treated}}{\left(\log \frac{N}{N_0}\right)_{control}} \qquad (A)$$

where N_0 and N are the initial and 24-hr cell concentrations, respectively.

Tumors

A *cis*-DDP-resistant line of L1210 leukemia (L1210/PDD) was obtained from Dr. J. Burchenal, Memorial Sloan-Kettering Institute (1), together with its parent line, which we designate

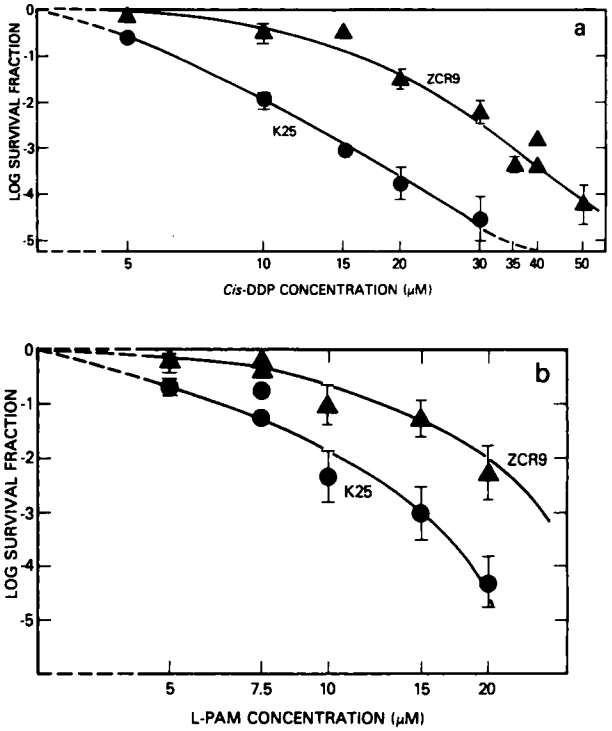

Chart 1. Colony survival of sensitive (line K25, ●) and resistant (line ZCR9, ▲) L1210 cells treated with various concentrations of *cis*-DDP or L-PAM for 1 hr. Double-logarithmic plots. *Points*, mean for ≥3 independent experiments. *Bars*, S.E.

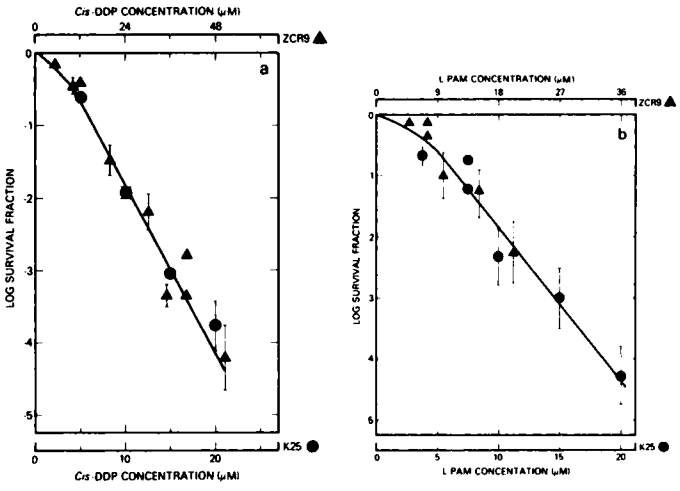

Chart 2. The same data as in Chart 1 plotted on linear concentration scales. The concentration scales used for the 2 cell lines differ according to the dose modification factor, determined as described in the text. *Symbols* are the same as in Chart 1. *Bars*, S.E.

L1210(MSKI). A L-PAM resistant line (L1210/PAM) originally derived by Dr. F. Schabel, Southern Research Institute, was obtained from Dr. D. Vistica, National Cancer Institute, together with its parent line which was obtained from the tumor bank of the National Cancer Institute and is designated L1210(NCI). The tumors were obtained in C57BL × DBA/2 F_1 (hereafter called BD2F_1) or DBA mice, but all experiments were carried out in male BD2F_1 mice.

The L1210/PDD and L1210(MSKI) lines were passaged weekly by i.p. injection of 10^6 cells. The mice bearing L1210/

PDD were treated with *cis*-DDP (4.5 mg/kg i.p.) on Days 1 and 5 following transplantation in order to maintain their resistance.

The L1210/PAM and L1210(NCI) lines were obtained as needed from Dr. D. Vistica, National Cancer Institute. The L1210/PAM line is passaged weekly by injection of 10^6 cells i.p., and resistance is maintained by treatment with L-PAM (7.5 mg/kg i.p.) on Day 2 following transplantation. The L1210(NCI) line is passaged weekly at 10^5 cells i.p.

Drug Treatment in Mice

For *in vivo* experiments, the clinical formulation of *cis*-DDP was used (10 mg *cis*-DDP, 100 mg mannitol, and 90 mg NaCl in 10 ml); L-PAM was freshly dissolved at 0.1 M in 10% 1 N HCl and 90% dimethyl sulfoxide (14).

Study Design

Mice bearing the L1210 cells to be used in an experiment were not treated with any drug. On Day 6 [L1210(MSKI) and L1210/PDD] or Day 7 [L1210(NCI) and L1210/PAM] following inoculation, cells were removed from a mouse, and an aliquot was inoculated into mice for survival studies. A second aliquot was placed into RPMI Tissue Culture Medium 1630 containing 20% fetal calf serum, 50 μM 2-mercaptoethanol, and either [2-^{14}C]thymidine (0.02 μCi/ml) or [*methyl*-^{3}H]thymidine (0.2 μCi/ml). The cells were adjusted to a concentration of 6 to 8 × 10^5/ml, incubated for 20 hr at 37°, and then washed twice and suspended in fresh RPMI Tissue Culture Medium 1630 containing 1% fetal calf serum. Equal numbers of oppositely labeled sensitive and resistant cells were mixed in order to assure reliable determination of differences between the tumor lines. The cell mixtures were treated with 20 μM *cis*-DDP or 20 μM L-PAM for 1 hr and then centrifuged, washed, and resuspended in RPMI Tissue Culture Medium 1630 containing 20% fetal calf serum. DNA cross-linking was determined by alkaline elution with and without proteinase K as described below (Assays 1 and 2; Ref. 7 and 9).

Thus, the cells for survival studies (Table 2) and those for cross-linking assays (Charts 8 and 9) were identical in each experiment. Groups of 5 to 6 mice were inoculated with 1 to 3 × 10^6 cells and monitored daily for survival. Increase in life span was calculated from the difference between the mean survival times of treated and untreated mice. Mean survival times of the controls were 7 to 8 days.

The biological behavior of the L1210(MSKI) and L1210(NCI) tumors were somewhat different. The volume of the ascites on the day of transplantation was greater in L1210(MSKI) than in L1210(NCI), and the L1210(MSKI) ascites tended to be less hemorrhagic. We have not assumed that L1210(MSKI) and L1210(NCI) are at all similar, but we have compared each only with the resistant line derived from it.

DNA Cross-Linking Studies

In order to optimize the precision of the comparison between 2 cell types, a double-labeling protocol was used. K25 cells were labeled overnight with [*methyl*-^{3}H]thymidine (specific activity, 20 Ci/mmol; 0.1 μCi/ml), and ZCR9 cells were labeled with [2-^{14}C]thymidine (specific activity, 51.4 mCi/mmol; 0.01 μCi/ml). (Reversing the labels did not significantly affect the results.) Labeled cells were washed twice and suspended in fresh medium containing 1% fetal calf serum. Equal numbers of the 2 cell types were then mixed. The cell mixture was treated with *cis*-DDP or L-PAM for 1 hr as described above.

DNA cross-linking was measured by alkaline elution as described previously (5, 8, 9, 15, 16). Approximately 10^6 cells were deposited on 25-mm diameter, 2-μm pore-size polyvinylchloride filters (Millipore Corp.; type BS). In Assays 1 and 3 (see below), filter holders with cylindrical solution reservoirs were used; whereas in Assay 2, a Swinnex (Millipore Corp.) filter holder was used (see Ref. 9). In Assays 1 and 3, the cells were lysed with 2% sodium dodecyl sulfate (BDH Biochemicals, Ltd., Poole, England)-0.1 M glycine-0.025 M EDTA (pH 10), and the detergent was washed out with 0.02 M EDTA (pH 10). Alkaline elution was then carried out by pumping a solution of approximately 0.1 M tetrapropylammonium hydroxide-0.02 M EDTA (pH 12.1) through the filter at 2 ml/hr, and fractions were collected for scintillation counting at 1.5-hr intervals for at least 15 hr. Radioactivity remaining in the filter and filter holder was also determined. In Assay 2, proteolytic digestion of the lysate was carried out by means of proteinase K (0.5 mg/ml) dissolved in the sodium dodecyl sulfate lysis solution. This proteinase solution was placed in the upper chamber of the Swinnex filter holder so as to fill the chamber, and the alkaline elution solution, containing also 0.1% sodium dodecyl sulfate, was overlayered in a syringe barrel mounted on the filter holder (9). The solutions were then pumped at the same rate as in Assays 1 and 3. The effective proteinase digestion time was estimated to be approximately 1 hr.

Three types of cross-linking assays were performed.

Assay 1. Cells were exposed to 300 R of X-ray at 0° in order to introduce a known frequency of DNA single-strand breaks. Alkaline elution conditions (described above) were such as to favor the adsorption of protein to the filters. This assay is thought to measure the combined effects of DNA-protein cross-links and interstrand cross-links. Cross-linking was quantitated in terms of cross-linking coefficient (K_c), defined as

$$K_c = \left(\frac{1 - r_0}{1 - r}\right)^{1/2} - 1 \qquad \text{(B)}$$

where r and r_0 are the fraction of the DNA retained on the filter for drug-treated and untreated cells, respectively. The end point for the determination of r and r_0 was usually at 10 hr of elution, but the value of K_c did not sensitively depend on the exact end point chosen.

Assay 2. Cells were exposed to 300 R of X-ray as in Assay 1, but the effects of DNA-protein cross-links were greatly reduced or eliminated by the use of proteinase K. This assay is thought to measure DNA interstrand cross-links. Cross-linking coefficient was calculated as above (Equation B). In both Assays 1 and 2, cross-linking coefficient has been found to be approximately proportional to the concentration of cross-linking drug used and is therefore thought to be a linear measure of cross-link frequencies (9). In this assay, the elimination of DNA-protein cross-linking and the linear dependence of cross-linking coefficient on drug dose allows the expression of interstrand cross-linking in rad equivalents by multiplying K_c by the dose of X-ray used in the assay (300 R in these experiments) (15, 17).

Assay 3. The assay is the same as Assay 1 except that the

cells received 3000 R of X-ray. The higher X-ray dose permits determination of the fraction of the DNA bound to protein and the calculation of DNA-protein cross-link frequencies (8, 12). DNA-protein cross-link frequency (p_x) was calculated from the formula

$$p_x = (1 - r)^{1/2} - (1 - r_0)^{1/2} p_b \qquad (C)$$

where r and r_0 are the fractions of DNA in the slow-eluting component (see Ref. 8) for drug-treated and untreated cells, respectively, and p_b is the frequency of DNA single-strand breaks produced by the X-ray exposure. The single-strand-break frequency produced by 3000 rads in L1210 cells is approximately 2.7 per million nucleotides. It is convenient to let p_b = 3000 rads, in which case the DNA-protein cross-link frequencies (p_x) are given in "rad equivalents." The absolute lesion frequencies can be estimated by multiplying the rad equivalents of p_x by 0.9×10^{-9} rad^{-1} nucleotide^{-1} (7, 8).

RESULTS

Sensitivity Differences between Parental and Resistant L1210 Cells in Culture. A resistant line (ZCR9) was selected from clones surviving treatment of the parental line (K25) with *cis*-DDP (see "Materials and Methods"). The sensitivities of the lines to *cis*-DDP and L-PAM were compared by determination of colony-forming ability and by measurements of cell proliferation in suspension culture.

The colony survival curves are shown in Chart 2 with concentration scales adjusted according to the dose modification factor determined as described in "Materials and Methods." When the results for the 2 cell lines are plotted in this way on the same graph, the points appear to fall on the same curve. Since the shapes of the survival curves are similar, the sensitivity difference between the 2 cell lines is indicated by the dose modification factor. The dose modification factor, *i.e.*, the drug concentration ratio (ZCR9/K25) that produced equal effects on the 2 cell lines, was 2.4 for *cis*-DDP (Charts 1*a* and 2*a*) and 1.8 for L-PAM (Charts 1*b* and 2*b*).

The measured colony survival was the same whether performed immediately after drug treatment or 24 hr later (data not shown). This rules out some possible artifacts stemming from the cloning procedure itself.

The cell proliferation assays, determined as increase in cell number over a 24-hr period, are shown in Chart 3 as a function of drug concentration. The dose modification factors, determined by the graph-sliding method described in "Materials and Methods," were 2.7 for *cis*-DDP (Chart 3*a*) and 1.6 for L-PAM (Chart 3*b*).

The drug effects (1-hr drug exposure) on cell proliferation over a more extended time period are shown in Chart 4. In these experiments, cells were maintained under relatively constant conditions for 6 days by daily centrifugation and resuspension in fresh medium. The proliferation of the sensitive line was markedly inhibited by both drugs, whereas the resistant lines treated with the same doses approached exponential growth.

Cross-Linking Differences in L1210 Cell Lines in Culture. DNA cross-linking was measured at various times following drug treatment by means of alkaline elution Assays 1 and 2 (see "Materials and Methods") using 300 R of X-ray to introduce single-strand breaks. Assay 1 (Chart 5, *upper panels*) measures the combined effect of DNA-protein cross-links and interstrand cross-links; Assay 2 uses proteinase K to reveal the effects of interstrand cross-links alone (Chart 5, *lower panels*). In the case of *cis*-DDP, DNA cross-linking measured by either method was uniformly less in ZCR9 than in K25 cells. There was no apparent difference between the 2 cell types in the kinetics of cross-link formation and removal. In the case of L-PAM, the cross-linking differences between the 2 cell lines in these experiments were less than those seen with *cis*-DDP. The half-times for cross-link removal appeared to be on the order of 10 hr for *cis*-DDP and 20 hr for L-PAM in both cell lines.

Cross-linking was quantitated in terms of cross-linking coefficient (defined in "Materials and Methods"), which has been found to be linearly related to drug concentrations, thus indicating that it is a linear measure of the frequencies of DNA-protein cross-links and of interstrand cross-links (9).

The relative cross-link frequencies in the 2 cell lines were more critically estimated by measurements as a function of drug concentration under conditions selected to maximize the contribution from each type of cross-link alone. DNA-protein cross-linking was measured immediately after drug treatment, since there is very little indication of interstrand cross-linking at this time (Ref. 15; Chart 5). In this case, DNA-protein cross-link frequency can be estimated from alkaline elution assays performed as in the experiments of Chart 5, except for the use of a 10-fold higher X-ray dose, namely, 3000 R (Assay 3; Ref. 8). The assay then separates protein-linked from free DNA single-strand segments. The theoretical and experimental basis of this procedure has been reported previously (8). The DNA-protein cross-link frequencies estimated by this method were linearly dependent on the concentration of *cis*-DDP (Chart 6) or L-PAM (Chart 7), and the ratios between the slopes of the lines are listed in Table 1.

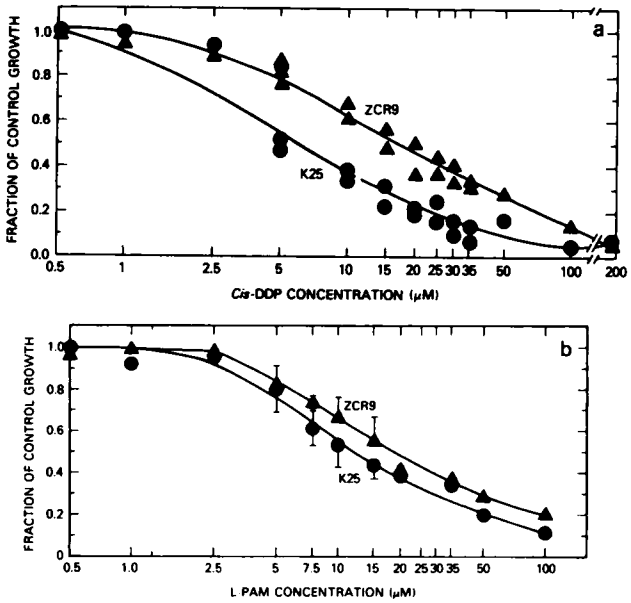

Chart 3. Concentration dependence of the effects of *cis*-DDP and L-PAM on proliferation of K25 and ZCR9 cells. Drug exposure time was 1 hr. *Ordinate*, increase in cell number in 24 hr as fraction of control growth, as described in "Materials and Methods." Control doubling times were 11.1 ± 0.5 hr for K25 cells and 13.1 ± 1.2 hr for ZCR9 cells. *Bars*, S.E.

The concentration dependence for DNA interstrand cross-linking was determined using the proteinase K method 6 hr after treatment, at which time delayed cross-link development was nearly complete. The results again showed linear concentration dependencies for *cis*-DDP (Chart 6) and L-PAM (Chart 7). The ratios of the slopes for K25 relative to ZCR9 cells are listed in Table 1.

The quantitative difference between the K25 and ZCR9 cells with regard to the 2 measures of cytotoxicity and the 2 DNA cross-linking effects are summarized in Table 1. For *cis*-DDP, all 4 dose modification ratios are comparable, indicating that all of the effects of the drug are altered by the same factor in the resistant (ZCR9) line. For L-PAM, the 2 cytotoxicity measures and the interstrand cross-linking assays gave comparable dose modification ratios. The assays for DNA-protein cross-linking, however, failed to show a difference between the 2 cell types.

Cross-Linking Differences in Sensitive and Resistant L1210 Ascites Tumor Lines. Two resistant L1210 tumor lines were obtained together with their respective parent L1210

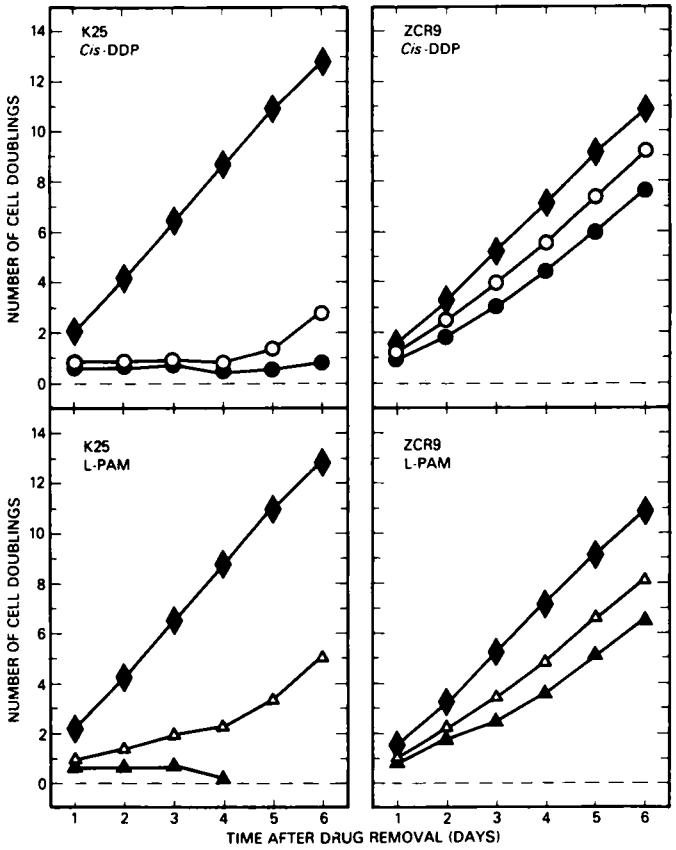

Chart 4. Effects of *cis*-DDP and L-PAM on extended proliferation of K25 and ZCR9 cells. ◆, no drug; ○, 10 μM *cis*-DDP; ●, 15 μM *cis*-DDP; △, 10 μM L-PAM; ▲, 15 μM L-PAM. Treatment times were 1 hr. Cells were centrifuged and resuspended in fresh medium daily.

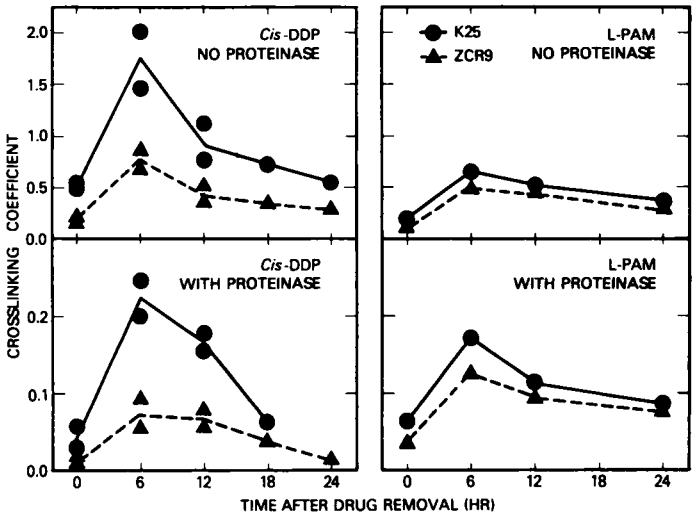

Chart 5. Cross-link formation and removal in K25 (●) and ZCR9 cells (▲). Cells were treated for 1 hr with either 20 μM *cis*-DDP or 20 μM L-PAM, and cross-linking was assessed at various times following drug removal. *Upper 2 panels*, total cross-linking; *lower 2 panels*, proteinase-resistant cross-linking. Cross-linking is quantitated by "cross-linking coefficient" (defined in "Materials and Methods"), a linear measure of cross-link frequencies.

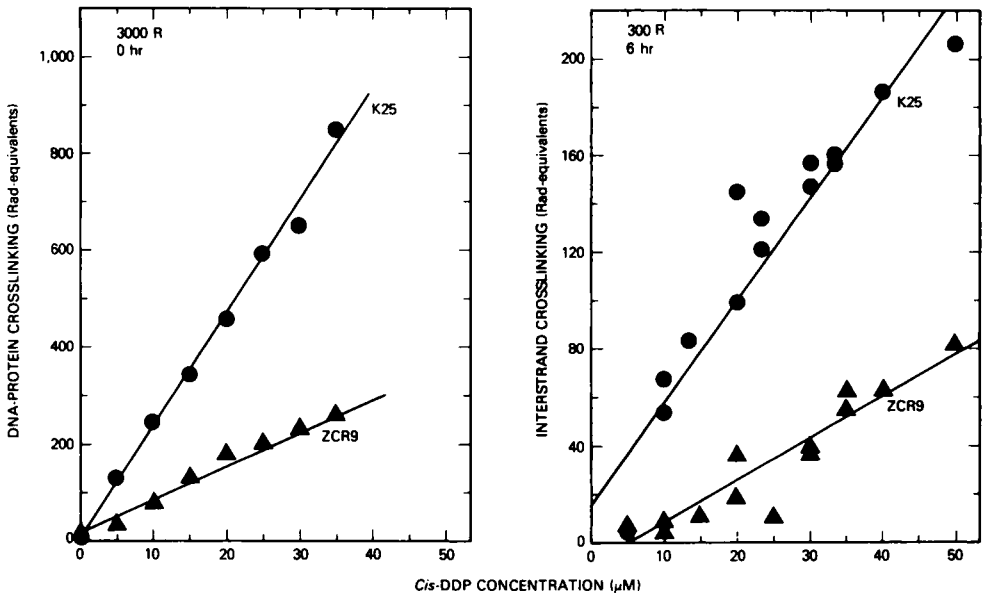

Chart 6. Concentration dependence of *cis*-DDP-induced DNA-protein cross-linking (*left*) and interstrand cross-linking (*right*) in K25 (●) and ZCR9 (▲) cells. Treatment times were 1 hr. DNA-protein cross-linking was determined immediately after treatment with *cis*-DDP, using 3000 R of X-ray in the assay; interstrand cross-linking was determined 6 hr after treatment with *cis*-DDP, using 300 R of X-ray in the assay (see text).

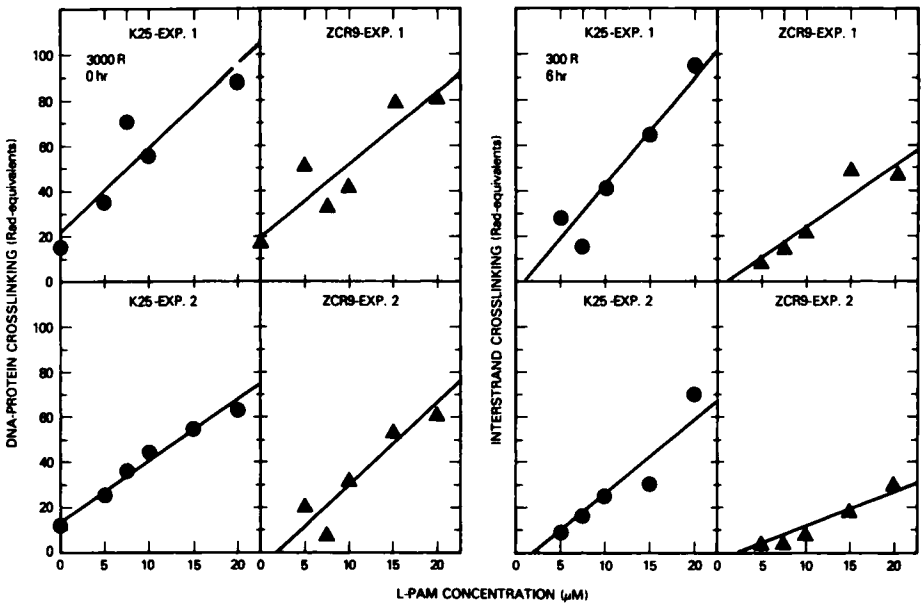

Chart 7. Concentration dependence of L-PAM induced DNA-protein cross-linking (*left*) and interstrand cross-linking (*right*) in K25 (●) and ZCR9 (▲) cells. *Symbols* are the same as in Chart 6. *Exp.*, experiment.

Table 1

Relative cytotoxicity and DNA cross-linking produced by cis-DDP and L-PAM in K25 and ZCR9 lines of L1210 cells; drug concentration ratios for equal effects in the 2 cell lines

Effect measured	*cis*-DDP	L-PAM
Colony survival[a]	2.4	1.8
Growth inhibition[a]	2.7	1.6
Interstrand cross-linking[b]	2.5	1.7, 2.2
DNA-protein cross-linking[b]	3.0	1.1, 0.8

[a] ZCR9/K25.
[b] K25/ZCR9. Ratios of slopes of cross-linking coefficient plotted against drug concentration, as in Charts 6 and 7.

Table 2

Increase in life span of mice bearing various L1210 tumor lines and treated with cis-DDP or L-PAM

Tumor line	% increase in mean life span					
	cis-DDP[a]			L-PAM[b]		
L1210(MSKI)	145	168	141	84	105	
L1210/PDD	11	11	10	61	92	
L1210(NCI)	73	28	97	86	86	116
L1210/PAM	4	5	9	22	35	18

[a] 4.5 mg/kg i.p. on Days 1, 5, 9, and 13 after inoculation of 1 to 3 × 10^6 cells i.p.
[b] 13 mg/kg i.p. on Day 1 after inoculation of 1 to 3 × 10^6 cells i.p.

strain (see "Materials and Methods"). The sensitivities of each sensitive/resistant pair of L1210 tumors to *cis*-DDP and L-PAM were determined concurrently with the cross-linking measurements (Table 2). The results were in accord with the findings of Burchenal *et al.* (1) and Schabel *et al.* (13); the L1210/PDD line was resistant to *cis*-DDP but not to L-PAM, and the L1210/PAM line was resistant both to L-PAM and to *cis*-DDP.

DNA cross-linking was determined at various times after *in vitro* treatment of the cells with 20 μM cis-DDP or 20 μM L-PAM for 1 hr (Charts 8 and 9). Assays were performed by alkaline elution with and without proteinase K. The assays without proteinase K (Charts 8 and 9, *upper panels*) are a combined measure of DNA-protein and interstrand cross-links; the assays with proteinase K (Charts 8 and 9, *lower panels*) measure interstrand cross-links alone. Each resistant line was compared directly in each assay with its corresponding control line as described in "Materials and Methods."

Chart 8, *left*, shows that the L1210/PDD line treated with *cis*-DDP exhibits much less cross-linking in either assay than does the control line. After treatment with L-PAM (Chart 8, *right*), however, the cross-linking results are similar in the 2 lines. This is in accord with the survival data (Table 2).

The corresponding results with the L1210/PAM line are shown in Chart 9. Treatment with L-PAM *in vitro* showed the expected reduction of cross-linking in the resistant line. However, the results with *cis*-DDP deviated from expectation; although L1210/PAM is highly resistant to *cis*-DDP *in vivo* (Table 2), *in vitro* treatment with this drug showed only small reductions in cross-linking relative to the control line.

The cross-linking results in all of the experiments are shown in Tables 3 and 4. Values listed on the same line in the tables are from the same experiment; values obtained within the same experiment were not subject to variability arising between different experiments. The cross-linking ratios shown are between resistant and parental cell pairs assayed within the same experiment. These results, summarized in Tables 5 and 6, represent in numerical form the data shown graphically in Charts 8 and 9.

The essential results are most easily seen in Table 6. Of the 2 parental L1210 lines used, the MSKI line appeared to be somewhat more sensitive than the NCI line to *cis*-DDP; however, this was not reflected by any difference in cross-linking. The strong resistance of L1210/PDD to *cis*-DDP and the greatly reduced cross-linking levels are evident. The lack of resistance of this line to L-PAM and the unchanged cross-linking levels also are clear. The resistance of L1210/PAM to L-PAM is less striking, and the reduction in cross-linking levels is also less than in the case of *cis*-DDP-treated L1210/PDD. These 3 cases show excellent correlation between *in vivo* sensitivity and *in vitro* cross-linking.

We again see, however, that the strong resistance of L1210/PAM to *cis*-DDP is accompanied by only a modest reduction in cross-linking levels [to approximately 66% of that in the parental line (Tables 5 and 6)].

The Question of Heterogeneous Cell Populations. We considered the possibility that this discrepancy could arise from a heterogeneous population of cells. It is conceivable that the

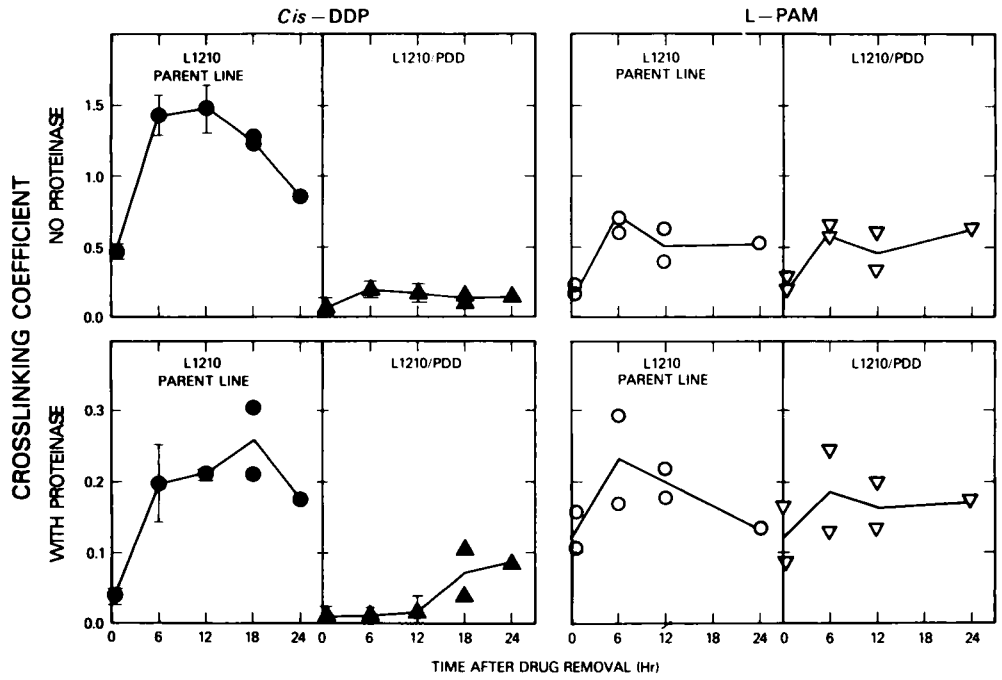

Chart 8. DNA cross-link formation and removal in L1210(MSKI) and L1210/PDD. Cells were treated for 1 hr with either 20 μM *cis*-DDP or 20 μM L-PAM, and cross-linking was assessed at various times following drug removal. *Upper panels*, total cross-linking; *lower panels*, proteinase-resistant cross-linking. *Bars*, S.E.

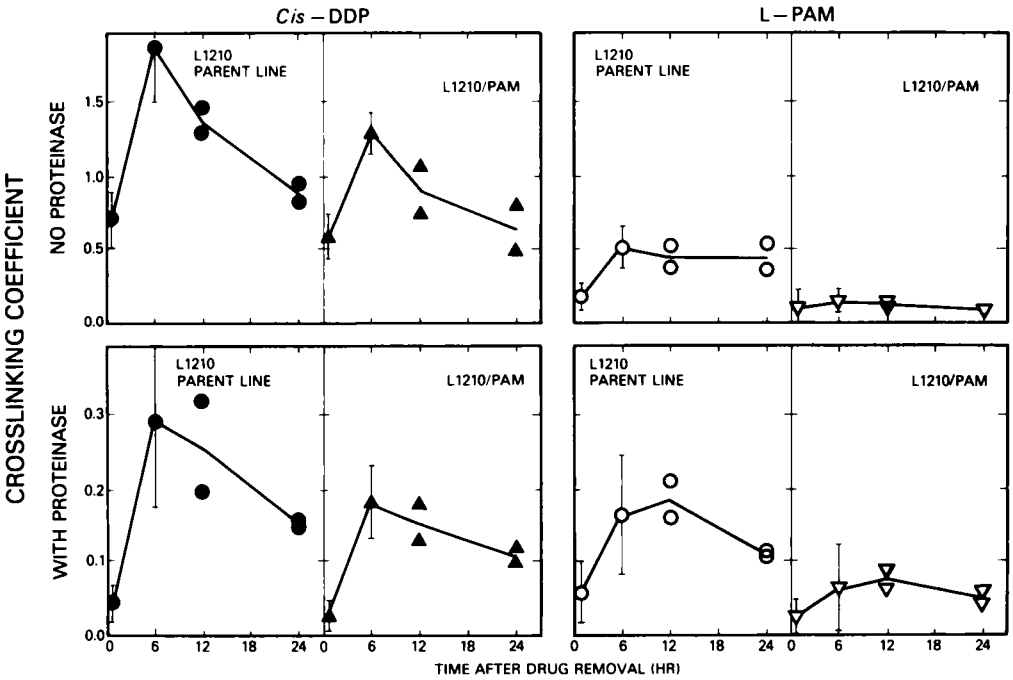

Chart 9. DNA cross-link formation and removal in L1210(NCI) and L1210/PAM. Drug treatment and experimental protocol is the same as in Chart 8. *Bars*, S.E.

L1210/PAM tumor consists of a major cell population that is sensitive to *cis*-DDP and exhibits high cross-linking plus a minor population that is cross-resistant to *cis*-DDP and is responsible for the poor survival of the animals. Although we have not definitely excluded this possibility, some preliminary results fail to support it.

An attempt was made to reduce or eliminate the hypothesized *cis*-DDP-sensitive component of the cell population by treating the tumor-bearing mice with *cis*-DDP. Mice bearing the L1210/PAM tumor were treated with *cis*-DDP 4.5 mg/kg on Days 1, 5, 9, and 13 following tumor implantation. This was done for 2 successive transplantation passages. During the next passage, the mice were not treated, and cells were removed for study of *in vitro* cross-linking by *cis*-DDP. The resistance of the cells to *cis*-DDP was confirmed by assay in mice. The results were similar to those obtained before (Chart 9; Table 5); the cross-linking ratio for resistant relative to sensitive cells 6 hr after *cis*-DDP was 0.75 in the assay without proteinase and 0.80 in the assay with proteinase. Hence, no support was obtained for the hypothetical heterogeneity of cell population. The possibil-

Table 3

DNA cross-linking following 1-hr treatment with 20 μM cis-DDP

Time after drug removal (hr)	Cross-linking coefficient											
	Without proteinase						With proteinase					
	L1210 (MSKI)	L1210/ PDD	Ratio[a]	L1210 (NCI)	L1210/ PAM	Ratio[a]	L1210 (MSKI)	L1210/ PDD	Ratio[a]	L1210 (NCI)	L1210/ PAM	Ratio[a]
0	0.42	0.05	0.12	0.78	0.78	1.00	0.04	0.00	0.00	0.04	0.04	1.00
	0.47	0.06	0.13	0.49	0.50	1.02	0.04	0.01	0.25	0.02	0.00	0.00
	0.48	0.05	0.10	0.85	0.51	0.60	0.05	0.01	0.20	0.07	0.04	0.57
6	1.49	0.23	0.15	1.90	1.40	0.74	0.24	0.02	0.08	0.20	0.13	0.65
	1.27	0.16	0.12	1.51	1.14	0.75	0.14	0.00	0.00	0.26	0.22	0.85
	1.52	0.20	0.13	2.27	1.38	0.61	0.23	0.02	0.09	0.43	0.21	0.49
12	1.66	0.16	0.10	1.47	1.08	0.73	0.21	−0.01	−0.05	0.20	0.13	0.65
	1.33	0.19	0.14	1.30	0.75	0.58	0.22	0.04	0.18	0.32	0.18	0.56
	1.41	0.17	0.12				0.20	0.01	0.05			
18	1.28	0.11	0.08				0.31	0.11	0.35			
	1.23	0.15	0.12				0.21	0.04	0.19			
24	0.85	0.15	0.18	0.96	0.82	0.85	0.18	0.08	0.44	0.16	0.12	0.75
				0.83	0.51	0.61				0.15	0.11	0.73

[a] Ratio of cross-linking coefficients within each experiment; derived line/parent line.

Table 4

DNA cross-linking following 1-hr treatment with 20 μM L-PAM

Time after drug removal (hr)	Cross-linking coefficient											
	Without proteinase						With proteinase					
	L1210 (MSKI)	L1210/ PDD	Ratio[a]	L1210 (NCI)	L1210/ PAM	Ratio[a]	L1210 (MSKI)	L1210/ PDD	Ratio[a]	L1210 (NCI)	L1210/ PAM	Ratio[a]
0	0.17	0.21	1.23	0.28	0.25	0.89	0.15	0.16	1.07	0.02	0.00	0.00
	0.17	0.17	1.00	0.14	0.06	0.43	0.11	0.08	0.73	0.07	0.03	0.43
				0.12	0.03	0.25	0.10	0.05	0.50			
6	0.67	0.59	0.88	0.37	0.08	0.22	0.17	0.13	0.76	0.12	0.03	0.25
	0.70	0.66	0.94	0.53	0.17	0.32	0.29	0.24	0.83	0.12	0.03	0.25
				0.66	0.23	0.35				0.26	0.14	0.54
12	0.43	0.36	0.84	0.53	0.16	0.30	0.18	0.14	0.78	0.16	0.06	0.38
	0.61	0.61	1.00	0.39	0.11	0.28	0.22	0.20	0.91	0.21	0.09	0.43
24	0.52	0.62	1.19	0.55	0.36	0.65	0.14	0.18	1.28	0.12	0.06	0.50
				0.37	0.09	0.24				0.11	0.04	0.36

[a] Ratio of cross-linking coefficients within each experiment; derived line/parent line.

Table 5

DNA cross-linking ratios of resistant relative to parent tumor lines

Determined 6 and 12 hr after treatment (from data in Tables 3 and 4).

Tumor comparison	*cis*-DDP		L-PAM	
	Without proteinase	With proteinase	Without proteinase	With proteinase
L1210/PDD / L1210(MSKI)	0.13 ± 0.02[a] (6)[b]	0.06 ± 0.08 (6)	0.92 ± 0.07 (4)	0.82 ± 0.07 (4)
L1210/PAM / L1210(NCI)	0.68 ± 0.08 (5)	0.64 ± 0.14 (5)	0.29 ± 0.05 (5)	0.37 ± 0.12 (5)

[a] Mean ± S.D.
[b] Number in parentheses, number of experiments.

Table 6

In vivo sensitivity and in vitro cross-linking by cis-DDP and L-PAM in various L1210 tumor lines (summary of data in Tables 2 to 4)

Tumor line	*cis*-DDP					L-PAM				
	% of ILS[a]	Cross-linking coefficient				% of ILS	Cross-linking coefficient			
		Without proteinase		With proteinase			Without proteinase		With proteinase	
		6 hr	12 hr	6 hr	12 hr		6 hr	12 hr	6 hr	12 hr
L1210(MSKI)	151	1.43	1.47	0.20	0.21	94	0.68	0.52	0.23	0.20
L1210/PDD	11	0.20	0.17	0.01	0.01	76	0.62	0.48	0.18	0.17
L1210(NCI)	66	1.89	1.38	0.30	0.26	96	0.52	0.46	0.17	0.18
L1210/PAM	6	1.31	0.92	0.19	0.16	25	0.16	0.14	0.07	0.08

[a] ILS, increase in life span.

ity is, however, not excluded that the sensitive population recovered during the last mouse passage.

In a second approach, L1210/PAM cells were cloned in soft agar in order to determine the properties of clones derived from single cells. So far, only one such clone has been studied. Colony survival measurements showed that this clone was resistant to *cis*-DDP, with a dose modification factor of 3.3 relative to a clone derived from the parent L1210(NCI) line. The corresponding cross-linking ratios (parental clone/resistant clone) were 1.8 (assay without proteinase) or 2.1 (assay with proteinase). Since these values are lower than the dose modification factor for colony survival, the cross-linking difference may not account for the survival difference. The difference between the cloned lines with regard to cross-linking, however, was greater than in the original lines from which the clones were derived. (The appropriate values for the original lines are obtained from Table 5, taking the reciprocals; thus, 1/0.68 = 1.47 for the assay without proteinase, and 1/0.64 = 1.56 for the assay with proteinase.) Thus, the resistant clone exhibited a greater reduction in cross-linking than did the original line, but this reduction was still inadequate to account for the magnitude of the resistance.

DISCUSSION

We have compared 3 sensitive/resistant pairs of L1210 lines in order to relate sensitivity to *cis*-DDP or L-PAM with the extent of DNA cross-linking.

In the first study, a cloned line of L1210 cells (ZCR9) derived by mutagenic treatment and selection for *cis*-DDP resistance was compared with its parent line (K25). In addition to its resistance to *cis*-DDP, the ZCR9 line exhibited resistance, although to a lesser degree, to L-PAM. This was a study of established cell lines in culture. The results, summarized in Table 1, support the proposition that DNA interstrand cross-linking measurements by alkaline elution can provide a quantitative measure of cytotoxic sensitivities of at least some cell types to bifunctional agents.

The best quantitative correlations of the sensitivities of these 2 cell lines to these 2 drugs were between the colony survival measurements and the measurements of DNA interstrand cross-linking (Table 1). The growth inhibition ratios were consistent with these values although quantitatively not as close. Since these growth inhibition assays (Chart 3) were done over 24 hr and the putative cytotoxic lesion produced by these drugs forms over 6 to 12 hr, accurate reflections of ultimate survival would be less likely in these assays than in colony survival assays (Charts 1 and 2) or longer growth studies (Chart 4). DNA-protein cross-linking, on the other hand, failed to correlate with cytotoxic sensitivity, in that L-PAM produced similar frequencies of these lesions in the 2 cell types, whereas the cytotoxic sensitivities differed significantly.

The second study utilized sensitive/resistant pairs of L1210 tumors in mice. Cells were removed from the peritoneal cavity and assayed for DNA cross-linking following drug treatment *in vitro*. The objective was to provide a first test of the feasibility of short-term *in vitro* assays to predict *in vivo* sensitivity. The results, summarized in Table 6, showed good correlation between DNA cross-linking and cytotoxicity in 3 cases: (*a*) a line highly resistant to *cis*-DDP showed greatly reduced cross-linking by *cis*-DDP; (*b*) the same line was fully sensitive to L-PAM and showed no reduction in cross-linking by L-PAM; and (*c*) a L-PAM-resistant line showed substantially reduced cross-linking by L-PAM.

In a fourth comparison, however, a possible discrepancy was found (Table 6). This was in an *in vivo* line which had been selected for resistance to L-PAM by serial treatment of L1210-bearing mice with L-PAM. The resulting L1210/PAM line was found to be cross-resistant to *cis*-DDP. Studies *in vitro* showed reduced DNA cross-linking (60 to 70% reduction) following L-PAM, as expected. But following *cis*-DDP, the L1210/PAM line exhibited only a 30 to 40% reduction in DNA cross-linking relative to the control line.

It is possible that this discrepancy might be due to a heterogeneous cell population, *i.e.*, a mixture of L-PAM-resistant cell types, some of which are sensitive and some of which are cross-resistant to *cis*-DDP. The presence of a minor component of *cis*-DDP-resistant cells could produce a small reduction in DNA cross-linking while producing a large reduction in life span. Two experimental tests, however, failed to support this possibility. (*a*) Treatment of the tumor with *cis*-DDP should have reduced or eliminated the *cis*-DDP-sensitive cells, but DNA cross-linking by *cis*-DDP remained the same as in the untreated tumor. (*b*) A cloned cell line derived from this tumor exhibited greater resistance to *cis*-DDP than could be quantitatively accounted for on the basis of reduced cross-linking. Further studies of cloned cell lines from this tumor are in progress.

A correlation between cytotoxic sensitivity and DNA interstrand cross-linking would be expected for several, although not all, of the possible cytotoxicity mechanisms or mechanisms of resistance. Cell lines may be supposed to differ, for example, with regard to drug uptake, intracellular drug activation or inactivation, or access of reactive drug to specific DNA sites. This group of hypotheses does not imply that the DNA cross-linking is causally related to cytotoxicity, although useful correlations may nevertheless exist between the magnitudes of the cross-linking and the cytotoxicity. The drug uptake factor cannot by itself account for the results with the ZCR9 line, because 2 chemically very different drugs (*cis*-DDP and L-PAM) are affected, and because, in the case of L-PAM, DNA-protein cross-linking in the 2 cell types was the same. For the same reasons, drug activation or inactivation cannot be the sole factor. Differences in DNA accessibility could, in principle, explain the data, because accessibility might be specific for drug and lesion type, but there is no theoretical basis for supposing that there are global differences in the chromatin of these cell types.

A second type of possible mechanism is that the resistant cells may more rapidly remove drug-DNA monoadducts before these monoadducts convert to cross-links. Such a mechanism was recently proposed to account for the greater sensitivity of a transformed compared with a normal human cell type to chloroethylnitrosoureas (3). *cis*-DDP and L-PAM, like chloroethylnitrosoureas, exhibit delayed formation of interstrand cross-links (Chart 5) so that prevention of these lesions by monoadduct removal is possible. This hypothesis, however, would not account for the reduced formation of DNA-protein cross-links by *cis*-DDP in ZCR9 cells, since these cross-links seem to be formed without detectable delay.

If monoadduct removal is the major determinant of interstrand cross-linking and survival, however, the removal must be specific for type of monoadduct, because in the human cell

system mentioned above, the relationship observed with *cis*-DDP was opposite to that observed with chloroethylnitrosoureas; *i.e.*, with *cis*-DDP, the normal cells were the more sensitive with regard to interstrand cross-linking and survival (4).

A third type of hypothesis is that the cells differ in ability to remove cross-links. However, our data do not support such a difference (Chart 5).

A fourth type of hypothesis is that cells may differ in ability to tolerate the presence of potentially lethal DNA lesions long enough to allow for their repair. This would tend to dissociate cytotoxic sensitivity from measured DNA damage.

Clearly, there is no single simple hypothesis that can account for all of these data. It is, of course, entirely possible that more than one of these factors contributes to the sensitivity difference, especially in the case of the ZCR9 line, which was derived by multiple cycles of mutagenic treatment and survival selection.

The present results demonstrate the feasibility of short-term *in vitro* measurements of DNA cross-linking and suggest that useful predictive correlations with tumor sensitivity are possible. These initial studies have been carried out in the particularly favorable case of extensively transplanted murine leukemias having high proliferative fractions. In clinical tumors, the circumstances would rarely be so favorable. Further work is required to determine the range of applicability of this approach.

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