[CANCER RESEARCH 41, 2791–2794, July 1981]
0008-5472/81/0041-0000$02.00

Differential Cytotoxicity and DNA Cross-Linking in Normal and Transformed Human Fibroblasts Treated with *cis*-Diamminedichloroplatinum(II)[1]

Leonard C. Erickson,[2] Leonard A. Zwelling, Jonathan M. Ducore, Nancy A. Sharkey, and Kurt W. Kohn

Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland 20205

ABSTRACT

DNA interstrand cross-linking had been found previously to correlate with differences in sensitivity among human cell strains treated with chloroethylnitrosoureas. These differences had been attributed to the presence or absence of a specific DNA repair mechanism. The current work addressed the question of whether another DNA cross-linking agent, *cis*-diamminedichloroplatinum(II) (*cis*-Pt), would exhibit analogous differences between cell types. A normal human embryo cell strain (IMR-90) was compared with an SV40-transformed line (VA-13). Interstrand cross-linking and DNA-protein cross-linking were assayed by alkaline elution. As in the case of chloroethylnitrosoureas, the cytotoxicity differences with *cis*-Pt correlated with differences in interstrand cross-linking. The relative sensitivity of the cell lines to *cis*-Pt, however, was reversed. Similar DNA-protein cross-linking levels in the two cell lines excluded a difference in *cis*-Pt uptake or intracellular metabolic drug activation or inactivation prior to DNA interaction. It was concluded that the DNA repair mechanism that prevents interstrand cross-linking by chloroethylnitrosoureas does not prevent interstrand cross-linking by *cis*-Pt. Interstrand cross-linking by *cis*-Pt may be prevented by an independent mechanism.

INTRODUCTION

The Pt(II) complexes are a new and effective group of antineoplastic agents. *cis*-Pt,[3] the most commonly used of these agents, is thought to exert its primary antitumor effect through bifunctional reactions with intracellular DNA in a manner similar to that for bifunctional nitrogen mustards (13, 17). We have recently found that the DNA interstrand cross-linking produced by Pt(II) complexes correlated with the cytotoxic potency of these platinum complexes in several cell systems (14, 15, 17, 18) and also with the sensitivity or resistance of several murine leukemia L1210 tumor lines in mice (18). This supported the concept that DNA interstrand cross-linking could be mechanistically related to the tumoricidal action of the *cis*-Pt.

In recent studies with another class of DNA cross-linking agents, the chloroethylnitrosoureas, we found a striking difference in the cytotoxicity and formation of DNA damage in a normal human embryo cells (WI-38 or IMR-90) as compared with an SV40-transformed human embryo cell line (VA-13) (1–3). The IMR-90 cells exhibited much less interstrand cross-linking and lower cytotoxicity in response to chloroethylnitrosoureas than did the VA-13 cells (1). DNA-protein cross-links, also produced by these drugs, however, were formed to a similar extent in the 2 cell types. It was therefore inferred that drug was able to enter and react within the 2 cell types to a similar extent. The difference in interstrand cross-linking was attributed to a difference in the ability of DNA repair mechanisms to remove drug-DNA monoadducts before their conversion to interstrand cross-links (1, 5).

We then posed the question of whether the differences between the 2 cell types would hold for other classes of DNA cross-linking agents. *cis*-Pt was suitable for this study because the types of DNA lesions formed by this drug and the kinetics of their formation are generally similar to the case of the chloroethylnitrosoureas. Both drug classes form interstrand cross-links with a delay of several hr and DNA-protein cross-links without appreciable delay (4, 7–9, 14). We find with *cis*-Pt that, as in the case of chloroethylnitrosoureas, there is a difference in cytotoxic sensitivity which correlates with interstrand cross-linking and not with DNA-protein cross-linking. Compared to the case of the chloroethylnitrosoureas, however, the relative sensitivity of the 2 cell types to *cis*-Pt is reversed.

MATERIALS AND METHODS

Cell Culture. IMR-90, a strain of normal human embryo cells, was obtained at passage 4 and population-doubling level 10 from Warren Nichols, Institute of Medical Research, Camden, N. J. The cells were grown to passage 8 and population-doubling level 14 and stored frozen in ampuls. Cells used in colony formation experiments were at passage 10; for other experiments, cells were at passages 10 to 30. The VA-13 line, an SV40 transformant derived from the normal human embryo cell strain, WI-38, was subcultured at low density in order to increase the plating efficiency of the line. Cells were cultured in Eagle's basal medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, and 50 μg of gentamicin per ml in an atmosphere of 7.5% CO_2 in air at 37°. The cultures were tested for *Mycoplasma* by Flow Laboratories, McLean, Va., and were free of contamination. Cells analyzed by alkaline elution were grown in the presence of [^{14}C]thymidine (0.02 μCi/ml; New England Nuclear, Boston, Mass.) for 24 hr, followed by removal of label and incubation in nonradioactive medium for an additional 24 hr before drug treatment.

Survival Studies. For colony formation assays, both cell types were used 2 days after plating 10^5 cells/25-sq cm flask, at which time the cells were in logarithmic growth phase. Single-cell suspensions were prepared by treating monolayers with 5% chicken serum, 0.1% trypsin, 25 units of collagenase

[1] This work was presented in part at the 71st Annual Meeting of the American Association for Cancer Research, San Diego, Calif. May 1980 (6).

[2] To whom requests for reprints should be addressed, at National Cancer Institute NIH, Building 37, Room 5D17, Bethesda, Md. 20205.

[3] The abbreviation used is: *cis*-Pt, *cis*-diamminedichloroplatinum(II).

Received August 5, 1980; accepted April 15, 1981.

per ml, and 0.02% EDTA in Hanks' balanced salt solution. This mixture produced higher plating efficiencies than did 0.25% trypsin or 0.05% trypsin/0.02% EDTA. For controls, 100 cells were plated per 100-mm-diameter plastic dish; for drug treatment, 1000 to 6000 cells were used. The dishes were incubated for 20 hr to allow the cells to attach. The cells were then exposed to drug for 2 hr. After 2 weeks of incubation in fresh media, the plates were fixed with methanol and stained with Giemsa, and colonies (at least 32 cells) were counted. This procedure avoids replating of cells after drug treatment.

In the cell proliferation studies, 5×10^5 IMR-90 or VA-13 cells were seeded in 25 sq cm flasks and incubated for 24 hr. The cells were then treated with drug for 2 hr in fresh medium supplemented also with 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cells were harvested after 3 days by trypsinization, and cell number was determined by means of an electronic cell counter.

Drug Treatment. The culture medium was replaced with fresh medium 30 min before drug treatment. *cis*-Pt was dissolved in Eagle's basal medium containing 10% fetal calf serum and 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid by stirring for 30 min at 37°. Appropriate volumes of 100 μM stock solution were added to cultures for 2 hr. Treatments were terminated by aspiration of the drug-containing medium and replacement with fresh culture medium.

Alkaline Elution. The alkaline elution procedure used in these experiments has been described in detail and reviewed recently (10, 12). Cells were analyzed by both the direct alkaline elution method and the proteinase modification. In the direct elution assay, 2 to 5×10^5 drug-treated ^{14}C-labeled cells were mixed with 5×10^5 ^{3}H-labeled mouse L1210 leukemia cells ([^{3}H]thymidine, 0.05 μCi/ml, New England Nuclear and 10^{-6} M unlabeled thymidine) in ice-cold 0.15 M NaCl/0.014 M KH_2PO_4/0.086 M K_2HPO_4, irradiated with 300 R of X-ray in the cold, and layered onto a 2-μm-pore-size polyvinyl chloride filter (type BSWP; Millipore Corp., Bedford, Mass.) using mild suction. (The ^{3}H-labeled L1210 cells served as internal standards in the assay.) The cells were immediately lysed with 5 ml of a solution containing 2% sodium lauryl sulfate, 0.1 M glycine, and 0.02 M EDTA, pH 10.0. Following lysis, the filter and lysate were washed with 3 ml of 0.02 M disodium EDTA/0.04 M NaOH, pH 10.0. Tetrapropylammonium hydroxide/0.02 M EDTA (pH 12.1) was pumped through the filter at 0.035 ml/min, and 1.5-hr fractions were collected. Fractions and filters were processed as described previously (10, 12). Radioactivity in each fraction was measured using a Packard 2450B liquid scintillation counter (^{14}C efficiency of 41% and ^{3}H efficiency of 16%).

The proteinase modification of the alkaline elution techniques minimizes the effect of DNA-protein cross-linking on the elution of DNA. The procedure was performed similarly to the direct assay except that 2-μm-pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, Calif.) were used and, following lysis, a solution containing 2% sodium lauryl sulfate, 0.1 M glycine, 0.02 M EDTA, 0.5 proteinase K,0.5 mg/ml, pH 10.0, was pumped through the filter for approximately 1 hr. The alkaline elution step was the same as in the direct assay except that 0.1% sodium dodecyl sulfate was added to the tetrapropylammonium/EDTA solution.

Apparent interstrand cross-link frequencies (in rad equivalents) were computed using the formula

$$(\sqrt{(1 - r_0)/(1 - r)} - 1) \times \text{(X-ray dose in rads)}$$

where r_0 and r are the fractions of the ^{3}H-labeled and ^{14}C-labeled DNA's remaining on the filter after approximately 10 hr of elution (11, 12).

DNA-protein cross-links were assayed by means of the high-dose X-ray alkaline elution method (11). A mixture of ^{14}C-labeled drug-treated cells and ^{3}H-labeled control cells was irradiated with 2000 R in the cold and subjected to alkaline elution from polyvinyl chloride filters without the use of proteinase K. The slow-eluting components were extrapolated to zero time, and DNA-protein cross-link frequencies were calculated by the formula

$$[(1 - r)^{-1/2} - (1 - r_0)^{-1/2}] \times \text{(X-ray dose in rads)}$$

where r and r_0 are the extrapolated fractions of slow-eluting DNA in drug-treated and control cells, respectively (11).

RESULTS

Cytotoxicity. The inhibiting effects of *cis*-Pt on cell proliferation and colony formation were studied using the same procedures as in our previous study of chloroethylnitrosoureas (1). The inhibition of cell proliferation by 2-hr *cis*-Pt treatments of IMR-90 and VA-13 cells is shown in Chart 1. The IMR-90 cells are seen to be more sensitive than were the VA-13 cells; the *cis*-Pt concentration ratio for 50% inhibition of VA-13/IMR-90 was approximately 5.

Colony survival assays also showed the IMR-90 cells to be much more sensitive than were VA-13 cells (Chart 2). In this experiment, cells were seeded at several densities and allowed to attach to the plates for 20 hr. The cells were then exposed to various concentrations of *cis*-Pt for 2 hr. The ratio of *cis*-Pt concentrations that reduced survival to 37% of control for VA-13 and IMR-90 was 6, and the ratio of D_0 values (ratio of slopes of the linear portions of the survival curves) was approximately 7.5.

DNA-Protein Cross-Linking. DNA-protein cross-linking was measured immediately after the 2-hr *cis*-Pt treatment, at which

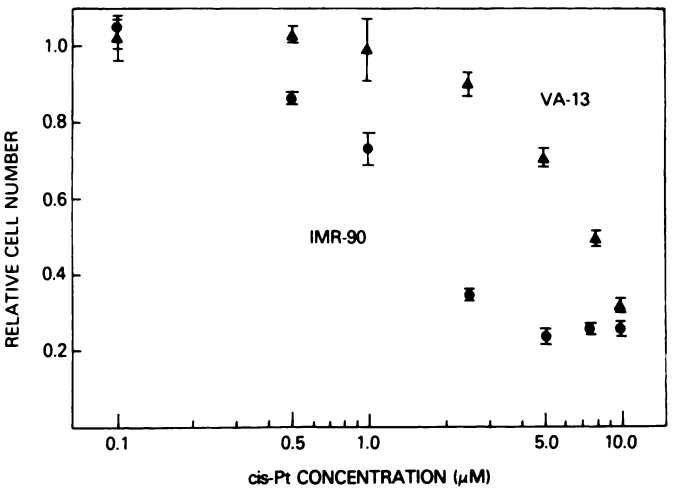

Chart 1. Effect of *cis*-Pt on proliferation of IMR-90 and VA-13 cells. Cells were treated with various concentrations of *cis*-Pt for 2 hr and then incubated for 3 days. Cell number in drug-treated cultures was determined relative to cell number in control cultures which completed approximately 3 doublings during this time.

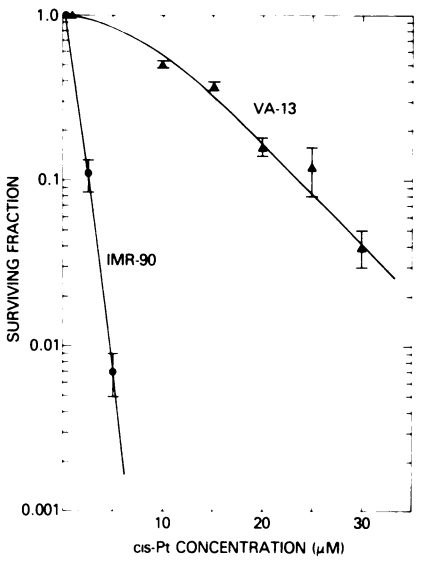

Chart 2. Survival of colony-forming ability of IMR-90 and VA-13 cells after treatment with various concentrations of *cis*-Pt for 2 hr.

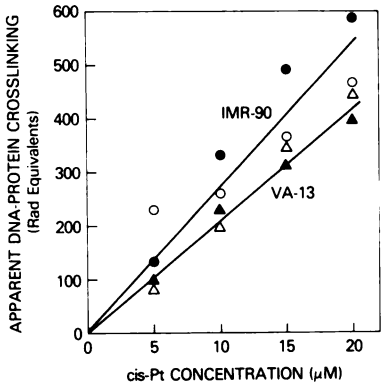

Chart 3. DNA-protein cross-linking in IMR-90 and VA-13 cells due to treatment with *cis*-Pt for 2 hr. Assays were performed immediately after removal of drug. Two independent experiments are shown: ●, ○, IMR-90 cells; ▲, △, VA-13 cells.

time little interstrand cross-linking had yet developed (Chart 6; Ref. 14). The high-X-ray-dose alkaline elution method (11) provided estimates of DNA-protein cross-link frequencies (Chart 3). DNA-protein cross-linking was proportional to *cis*-Pt concentration. The ratio of DNA-protein cross-link frequencies for IMR-90 relative to VA-13 cells was only 1.3. Because of the proportionality of DNA-protein cross-linking to drug concentration, this ratio is equal to the ratio of drug concentrations (VA-13/IMR-90) which produce equal DNA-protein cross-linking. This 1.3 ratio is much too small to explain the dose ratios of 5 or more for cytotoxicity.

DNA Interstrand Cross-Linking. Chart 4 shows alkaline elution assays for DNA interstrand cross-linking in IMR-90 and VA-13 cells exposed to several *cis*-Pt concentrations. Cells were treated with the indicated doses for 2 hr; the medium was then replaced, and the cells were incubated for an additional 12 hr. Alkaline elution assays were performed using the proteinase K method in order to eliminate DNA-protein cross-links. In both cell lines, DNA elution is seen to decrease with increasing *cis*-Pt concentration, indicating dose-dependent interstrand cross-linking. The magnitude of the cross-linking effect in the IMR-90 cells is much greater than in the VA-13 cells (Chart 4).

Interstrand cross-linking was quantitated as described under "Materials and Methods" and plotted against *cis*-Pt concentration (Chart 5) and against time following treatment (Chart 6). The calculated cross-linking values were proportional to *cis*-Pt concentration (Chart 5). Proportionality to drug concentration is the main basis for the assumption that the cross-linking values calculated in this way constitute a linear measure of the cross-link frequencies. The ratio of interstrand cross-linking in

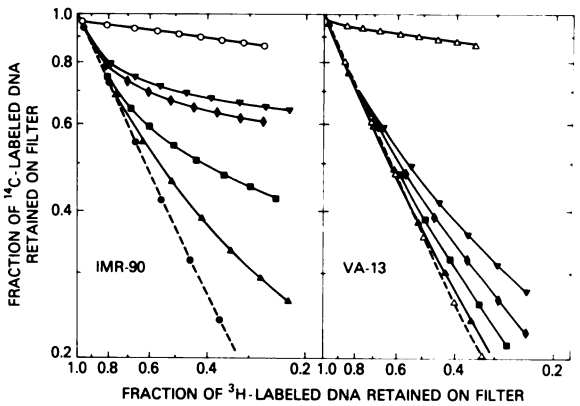

Chart 4. Alkaline elution assays for DNA interstrand cross-linking in IMR-90 and VA-13 cells exposed to various concentrations of *cis*-Pt for 2 hr and then incubated in the absence of drug for 12 hr. *cis*-Pt concentrations (μM): ●, △, zero; ▲, 5; ■, 10; ◆, 15; ▼, 20; ○, △, no drug and no X-ray.

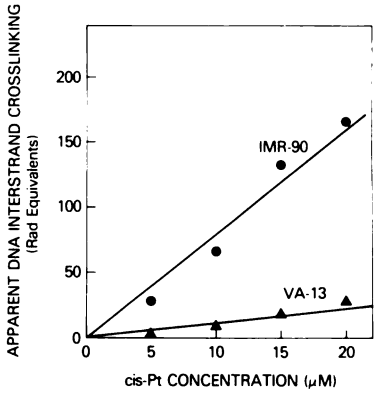

Chart 5. DNA interstrand cross-linking in IMR-90 and VA-13 cells following a 2-hr exposure to *cis*-Pt and incubation for 12 hr in the absence of drug. Values are calculated from the experiment shown in Chart 4.

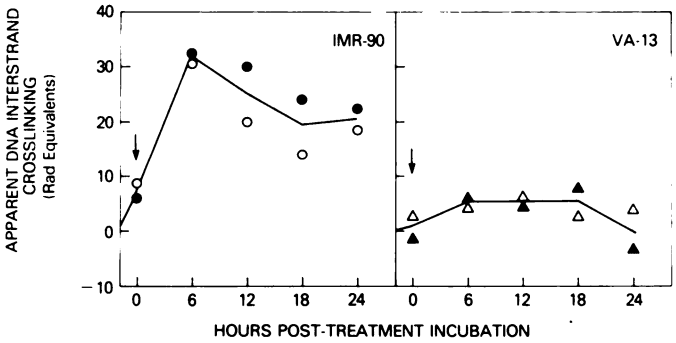

Chart 6. DNA interstrand cross-linking in IMR-90 and VA-13 cells as a function of time after exposure of the cells to 7.5 μM *cis*-Pt for 2 hr and incubated for the indicated times without drugs. *Symbols* indicate independent determinations. *Arrows*, time of drug removal.

IMR-90 relative to VA-13 cells, determined by the ratio of slopes in Chart 5, was 6.3.

The kinetics of interstrand cross-linking following 2-hr treatments with 7.5 μM *cis*-Pt is shown in Chart 6. Interstrand cross-linking in IMR-90 cells peaked at approximately 6 hr and declined only to a limited extent over the subsequent 18 hr. Interstrand cross-linking in VA-13 cells was very low throughout the 24-hr period of observation. It can be seen from Chart 6 that the ratio of 6.3 for interstrand cross-linking at 12 hr after drug, obtained in Chart 5, also holds true at 6 hr.

DISCUSSION

We have compared a normal human embryo cell strain (IMR-90) with an SV40-transformed human embryo cell line (VA-13) with regard to sensitivity to the cytotoxic and DNA cross-linking effects of *cis*-Pt. Cytotoxicity, assayed by inhibition of cell proliferation and by inhibition of colony-forming ability, showed that IMR-90 cells were 5 to 8 times more sensitive than were VA-13 cells; *i.e.*, 5 to 8 times the drug concentration was required to produce a comparable inhibition of VA-13 cells as compared to IMR-90 cells (Charts 1 and 2).

This sensitivity difference conformed with a 6-fold greater efficiency for interstrand cross-link formation in IMR-90 as opposed to VA-13 cells (Charts 5 and 6). Interstrand cross-linking was gauged 6 to 12 hr after drug exposure, at which time interstrand cross-linking was near maximal.

The agreement between the VA-13/IMR-90 dose modification ratios for cytotoxicity and interstrand cross-linking suggests a mechanistic relation between these events. One class of possibilities is that the cells differ with regard to drug uptake or in ability to activate or inactivate the drug intracellularly prior to interacting with target DNA molecules. These possibilities, however, were ruled out by the finding that the 2 cell types differed relatively little in the formation of DNA-protein cross-links (Chart 3). The observed ratio of 1.3 (IMR-90/VA-13) is not sufficient to explain the 5- to 8-fold difference in cytotoxicity.

In other work, however, we have found that certain *cis*-Pt-resistant mouse leukemia L1210 cells exhibit reductions in interstrand cross-linking and DNA-protein cross-linking, both in proportion to the dose modification ratio for cytotoxicity (18). This type of result suggests that the resistance of that L1210 line is due to reduced ability to transport *cis*-Pt into the cell or increased ability to inactivate *cis*-Pt within the cell resulting in lower levels of platinum-DNA interaction.

In contrast to the delay of 4 to 6 hr in interstrand cross-link formation, the DNA-protein cross-links form without appreciable delay. It is therefore possible that the cells differ in ability to repair platinum-DNA monoadducts before these are converted to interstrand cross-links. We have proposed this type of mechanism to explain analogous results with chloroethylnitrosoureas (1, 5). With both drugs, DNA-protein cross-linking is rapid and may not allow enough time for monoadduct repair to be effective in preventing this type of cross-linking.

The most interesting aspect of our results, however, is that, although *cis*-Pt and chloroethylnitrosoureas show similar DNA cross-linking characteristics and similar correlations with cytotoxicity, the relative sensitivity of the 2 cell types studied was reversed. Whereas the transformed line was more sensitive to chloroethylnitrosoureas than was the normal cell strain (1), the reverse was true for *cis*-Pt. Our results with chloroethylnitrosoureas suggested that the greater sensitivity of the transformed cells was due to a deficiency in a repair mechanism that removes certain DNA monoadducts (1, 5). Carrying this logic over to the case of *cis*-Pt would suggest the unexpected possibility that the transformed cells are more capable than the normal cells in removing platinum-DNA monoadducts. Rather than monoadduct repair, however, the relevant event in the case of *cis*-Pt could be DNA-platinum monoadduct inactivation. This could occur by the reaction of a sulfur-containing compound with the second negative site of a singly DNA-bound platinum complex (16). The VA-13 cells may contain a higher concentration of such platinum-active compounds than do IMR-90 cells. In any case, our results indicate that differential cytotoxicity that is related to interstrand cross-linking can be drug specific.

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