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# Cisplatin–DNA Damage and Repair in Peripheral Blood Leukocytes *in Vivo* and *in Vitro*

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**We have extended our studies on the relationship between cisplatin/carboplatin-induced DNA damage in readily accessible tissue(s) and clinical response to therapy. Such an approach may assist in the study of cancer drug resistance and in establishing parameters for assessing human populations for sensitivity to DNA damaging agents in the environment. Platinum–DNA adduct levels were measured by atomic absorbance spectrometry. DNA repair capacity was assessed in human T-lymphocytes by the ability to repair cisplatin lesions in cellular DNA or in transfected plasmid DNA. In a "blinded" study of 21 patients receiving combination cisplatin/carboplatin drug therapy, there was a direct relationship between DNA damage in leukocytes and disease response (summary two-sided $p = 0.00011$). The cohort of patients had 15 different tumor types, suggesting that blood tissue and tumor tissue of an individual may process platinum–DNA damage similarly regardless of the tissue of origin of the tumor. In leukocytes *in vivo*, persistence and accumulation were prominent features of the cisplatin–DNA adduct profile. Functional DNA repair capacity has been studied in eight human leukocyte cell lines *in vitro* (three, T-cells; three, B-cells; one, monocytic; one, promyelocytic), using a host cell reactivation assay with cisplatin-damaged pRSVcat. In the three T cell lines studied, host cell reactivation efficiency was directly related to the cells' abilities to repair cisplatin-damaged cellular DNA (correlation coefficient = 0.993). These data suggest that blood tissue and tumor tissue may process platinum–DNA adducts similarly on a molecular level and that human T lymphocytes are a suitable target for a prospective study of the impact of DNA repair on clinical response to platinum compounds.**

## Introduction

Cisplatin and carboplatin are anticancer therapeutic agents that are carcinogenic and mutagenic (*1,2*). These drugs may induce a number of different cellular effects through many different mechanisms (*1,3*); however, direct covalent binding to DNA with subsequent disruption of normal DNA function is considered to be the primary mechanism through which this class of agents effects its carcinogenic and mutagenic behavior (*1–3*). Whereas many DNA damaging agents may induce deleterious health effects in human populations (*4*), few agents are as amenable to study as platinum compounds: because controlled, precise doses can be given; there are negligible "background" levels of these agents in the environment; individuals exposed to the drugs are monitored for defined clinical end points; and the methods for assessing platinum–DNA damage levels are well established.

Work has been performed by three groups to assess human patient populations for platinum–DNA damage. The most extensive set of studies to date has been performed by Reed and Poirier in patients with ovarian or testicular cancer (*5–8*). In these studies, which involve more than 100 patients, the relationship of platinum–DNA damage level in peripheral blood leukocytes is consistently and directly related to response for the two diseases. Further, tumor and bone marrow tissue obtained at autopsy from patients who had been treated with platinum compounds have similar levels of platinum–DNA adducts (*6,9,10*). These studies have received some criticism because of the use of polyclonal antisera to detect adducts in an enzyme-linked immunosorbent assay (ELISA), the results of which do not correlate well with platinum–DNA damage levels measued by atomic absorbance spectrometry (AAS) (*11*).

Den Engelse and colleagues have used immunodensitometry to study adduct levels in buccal mucosa cells from patients receiving cisplatin and carboplatin therapy (*12*). They found a direct relationship between adduct level

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and disease response in their patient cohort, although the relationship was not statistically significant. This method has also been criticized because of the lack of a one-to-one relationship between what is measured in this assay and what is measured by AAS.

Fichtinger-Schepman and colleagues have used antisera directed against isolated platinum–DNA base adducts and have also reported a trend to higher adduct levels in patients who respond to therapy (*13*). They consider that their assay is advantageous because of the close (but not one-to-one) relationship between the measures of their assay and of AAS; however, this conclusion is based on the study of a very small number of samples (*14*). Recent reports by Reed and colleagues suggest that current AAS technology may now be used routinely for studies of platinum–DNA binding following exposure to therapeutic levels of the drugs (*9,15,16*).

Because of the importance of the questions raised by studies of this kind, we have conducted two types of studies in fresh human tissues. In the first, we used AAS to study platinum–DNA damage in peripheral blood leukocytes from a cohort of patients with 15 different tumor types who had received cisplatin and carboplatin in a phase-I clinical study (*12*). We considered that if platinum–DNA adduct levels were directly related to clinical response in this mixed cohort of patients, it would suggest a parallel between blood tissue and tumor tissue in the processing of platinum–DNA damage, regardless of tumor type.

In the second set of studies, we assessed the usefulness of a functional cisplatin–DNA adduct repair assay in human leukocyte cell lines. This host-cell reactivation assay assesses a cell's ability to express the chloramphenicol acetyltransferase gene as a function of the amount of cisplatin damage introduced into a plasmid that is transfected into those cells. The assay accurately reflects the relative cisplatin–DNA adduct repair capability of human ovarian cancer cells (*16,17*). The possible relationships between DNA damage and repair in malignant and nonmalignant cells are discussed.

# Methods

## Patients Studied and Sample Collection

Twenty-one adult patients with histological documentation of cancer were treated in an approved experimental clinical study of cisplatin and carboplatin given in combination. The study was conducted at the Norris Cancer Center of the University of Southern California (USC) School of Medicine, Los Angeles, California. Carboplatin was given as a 30-min infusion on day 1 and cisplatin as a 30-min infusion on day 3 of 28-day treatment cycles (both drugs were obtained from commercial sources). There were 15 different histological types of malignancy in this cohort, as listed in Table 1. Details of patient eligibility and clinical results of the study have been reported elsewhere (*12*). Peripheral blood samples were obtained at USC by venipuncture, and buffy coats were prepared as described previously (*5–8*).

**Table 1. Histological types of tumors seen in a cohort of 21 patients treated with carboplatin and cisplatin therapy.**

| Tumor types of responders | Tumor types of nonresponders |
|---|---|
| Breast adenocarcinoma | Colon adenocarcinoma |
| Buccal mucosa, squamous-cell carcinoma | Liver adenocarcinoma |
| Esophagus, squamous-cell carcinoma | Lung adenocarcinoma |
| Ovary, adenocarcinoma | Lung large-cell carcinoma |
| Pleural mesothelioma (two responders) | Lung small-cell carcinoma |
| | Melanoma |
| | Pancreas, adenocarcinoma |
| | Parotid, adenoid cystic |
| | Parotid, undifferentiated |
| | Uterus, leiomyosarcoma |

Blood samples were obtained from all patients at the same times relative to therapy: *a*) the morning of the first dose of therapy, but prior to any therapy (C1D1); *b*) 24 hr after the first dose of therapy (C1D2); *c*) 24 hr after the second dose of therapy (C1D4); *d*) the morning of the first dose of the second cycle of therapy, but prior to that dose (C2D1); *e*) 24 hr after the first dose of the second cycle (C2D2), i.e., the third dose of therapy; and *f*) 24 hr after the second dose of the second cycle (C2D4), i.e., the fourth dose of therapy.

The study was designed to study platinum–DNA adducts in peripheral blood cells in a "blinded" fashion. Therefore, all blood samples were obtained as described, coded, and shipped on dry ice to the Medicine Branch of the National Cancer Institute (NCI), Bethesda, Maryland. After arrival, they were stored at −20°C until isolation of DNA. DNA was isolated (see below) and adduct levels measured in the absence of any information about patients.

## Isolation of DNA for Measurement of Platinum–DNA Adducts

DNA was isolated in the same way from buffy coats from patients and from cell pellets *in vitro*. Cesium chloride bouyant density gradient centrifugation was performed on each sample as previously described, yielding DNA that was > 99% free of contamination (*18*).

## Cell Lines Studied and Culture Conditions

Eight human leukocyte cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in culture according to specific recommendations of the ATCC. They comprised three T lymphocyte cell lines (HuT 78, H9, and MOLT-4), three B lymphocyte cell lines (WIL2-NS, SKW 6.4, and RPMI 1788), the monocyte cell line THP-1, and the promyelocytic cell line HL-60. All cell lines were assayed for sensitivity to cisplatin by measuring inhibition of growth following 3-day exposures to cisplatin at 0.5–100 μM. Cells were seeded at an initial cell density of $5 \times 10^4$ cells/mL.

## pRSVcat Transfection Assay

The plasmid pRSVcat was propagated and amplified in *Escherichia coli* and purified using cesium chloride/

ethidium bromide density gradient centrifugation of bacterial lysates, as previously described (*16,19*). Aliquots of plasmid were modified with cisplatin in the presence of sodium perchlorate, pH 5.5, as previously described (*16,19*). pRSVcat was transfected into cells by electroporation under conditions found optimal for undamaged plasmid (215 V, 800 μF) (*19*), using $5 \times 10^6$ cells in 0.5 mL of medium. Chloramphenicol acetyltransferase activity was measured 24 hr later and, in some experiments, 48 hr later.

## Data Analyses

Patients were grouped according to clinical response to therapy. Those who had a > 50% decrease in tumor mass were designated as responders, and those who experienced a < 50% decrease in tumor mass were designated as nonresponders. Comparison of adduct levels between responders and nonresponders on each day was based on the Wilcoxon rank sum test (*20*). A summary comparison of adduct levels between responders and nonresponders across all 4 days was performed using the stratified Wilcoxon rank sum test (*20*).

For studies *in vitro*, linear regression analyses were performed using the Cricket Graph software package and a Macintosh II computer (Apple, Inc.).

# Results

## Adduct Levels in Peripheral Blood Leukocytes and Disease Response

Previous studies showed a direct relationship between DNA adduct levels measured in ELISA and disease response in ovarian cancer (*6,8*) and in testicular cancer (*5*). One shortcoming of those studies is that blood samples for determination of adducts were drawn at the convenience of the patient and the physician, and not necessarily in a systematic fashion, as was done in our study. We attempted to collect all designated samples from all patients. Some patients could not cooperate in sample collection; in other cases, the collected sample contained such a small number of nucleated blood cells that the quantity of DNA obtained was insufficient to assay the adduct level.

Figure 1 shows the results obtained for patients in this study at C1D2, C1D4, C2D2 and C2D4. Patients who responded to therapy had consistently higher levels of platinum–DNA damage than patients who did not respond. Table 2 shows the same data in tabular form, as well as the results of statistical analyses of the differences between responders and nonresponders. For C1D2 and C2D4, the differences were statistically significant. For C1D4 and C2D2, the differences showed a strong trend toward significance. We next assessed the collective trend in the pattern across all 4 days studied and found that the pattern of responders with higher adduct levels than nonresponders on each of the days studied gives a summary two-sided $p = 0.00011$ in this cohort of 21 patients with 15 different tumor types.

## Adduct Accumulation and Persistence in Peripheral Blood Leukocytes

Although our efforts were focused on collecting all designated blood samples from cycle 1 of therapy, we were able to collect adequate blood samples for adduct measurement on days 1, 2, and 4 of cycles 1 and 2 for only four individuals (i.e., adequate samples collected on 6 days studied). The mean values from these determinations are shown in Figure 2.

The morning before the first dose of carboplatin (C1D1), these four patients showed no detectable level of platinum–DNA adducts in leukocyte DNA. On C1D2 (24 hr after carboplatin), measurable levels of adduct could be assessed. On C1D4 (24 hr after cisplatin), the average DNA adduct level was greater than 40 fmole/μg DNA. On C2D1 (the morning before the second carboplatin dose and 25 days after the most recent dose of platinum), an average of 12 mole adduct remained on cellular DNA in these patients, suggesting that 30% of the adduct load seen on C1D4 persisted for 25 days after the most recent exposure to platinum.

On C2D2 (24 hr after the second carboplatin dose), the incremental rise in adduct levels was about the same as that seen during the same period in cycle 1. On C2D4, however, there was a disproportionate rise in adduct levels as a result of the D3 cisplatin dose. The incremental increase in measured adduct level from day 2 to day 4 was 4-fold greater in cycle 2 than in cycle 1. It is unclear whether this result is due simply to an increase in the amount of initial DNA damage, to a partial inactivation of DNA repair enzymes from a direct covalent attack of cisplatin on proteins, or to some combination thereof.

These data show that in tissues of living human patients platinum–DNA adducts persist for periods of ≥ 25 days and that adduct accumulation may result from this persistence and/or as a result of other processes. This persistence reflects the amount of platinum–DNA damage that is not readily repaired by these cells.

## DNA Repair of Cistplatin-damaged Plasmid in Eight Human Leukocyte Cell Lines

Since DNA repair can vary markedly between individuals and from tissue to tissue within an individual, we became concerned with developing a way to study DNA repair for the purpose of comparing one individual to another. Since peripheral blood appears to be a suitable surrogate marker for studying platinum–DNA binding effects, we have begun to address the question of whether peripheral blood can serve as the basis for interindividual comparisons of DNA repair efficiently.

As reported previously, we developed a rapid single-vial transfection assay to study cellular repair of cisplatin-modified pRSVcat plasmid in the human H9 T lymphocyte cell line (*19*). Using the parameters of this assay, we transfected plasmid into each of eight human leukocyte cell lines. As shown in Figure 3, the assay was equally effective in transferring plasmid into each of the eight cell lines studied. In each case, the amount of intracellular

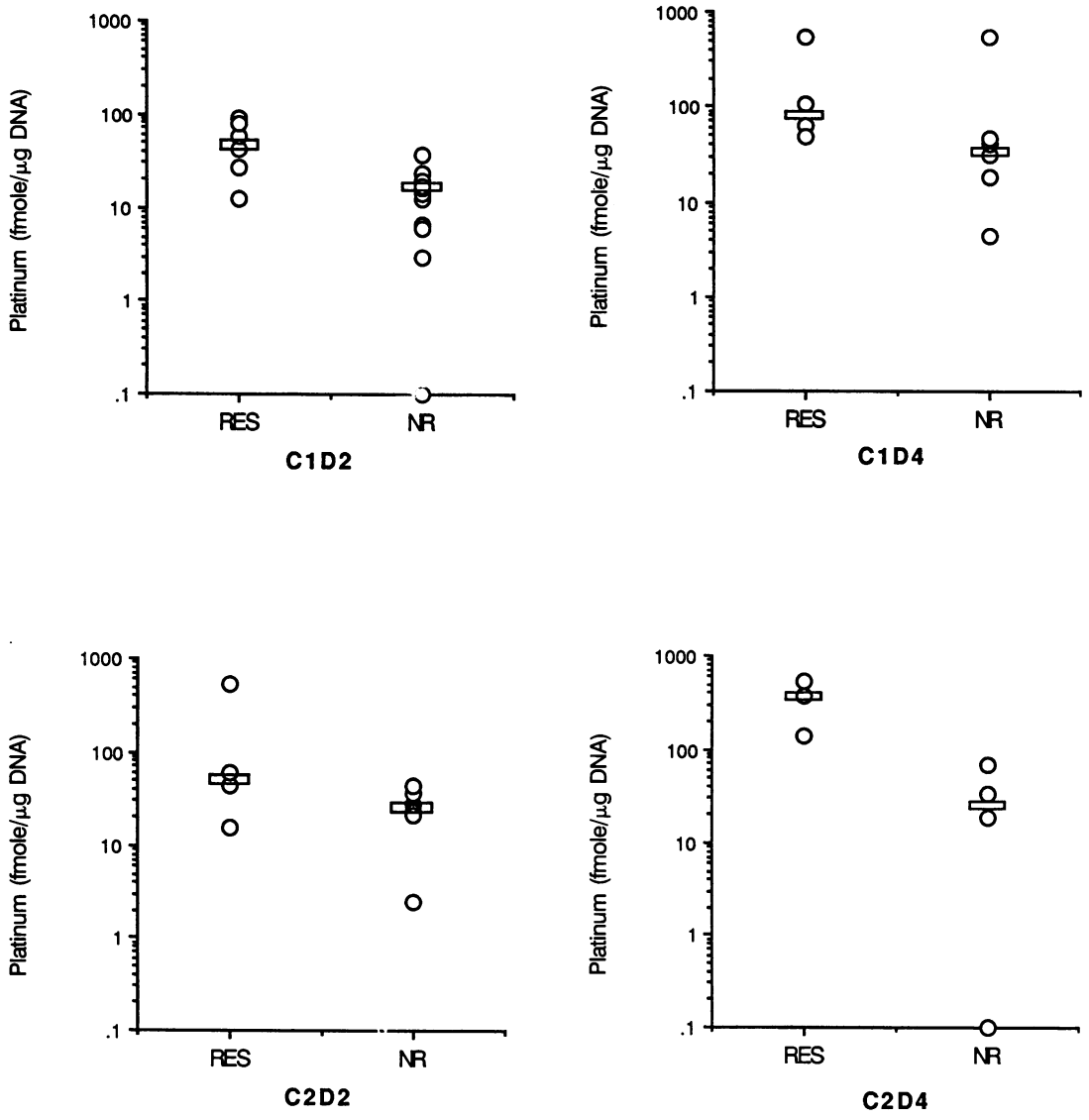


FIGURE 1. Platinum–DNA adduct levels (measured by atomic absorption spectrometry) are plotted for responders (RES) and nonresponders (NR) for each of the 4 days following chemotherapy on the first two cycles of therapy. C1D2 samples were taken 24 hr after the cycle 1 carboplatin dose; C1D4 samples were taken 24 hr after the cycle 1 cisplatin dose; C2D2, 24 hr after the cycle 2 carboplatin dose; and C2D4, 24 hr after the cycle 2 cisplatin dose. Horizontal bars represent median values in each group of data points.

**Table 2. Platinum–DNA adduct levels and disease response in 21 patients treated with cisplatin and carboplatin (not all patients had samples that could be evaluated on all days).**

| | Median adduct levels[a] | | |
|---|---|---|---|
| Cycle/day | Responders | Nonresponders | $p$-Value[b] |
| C1D2 | 49.2 (6) | 14.4 (11) | 0.0071 |
| C1D4 | 105.0 (5) | 40.0 (8) | 0.097 |
| C2D2 | 51.6 (4) | 26.0 (8) | 0.198 |
| C2D4 | 367.7 (3) | 26.3 (5) | 0.036 |
| | | Summary two-sided $p$ = 0.00011 | |

[a]Number of patients studied in parentheses.
[b]Two-sided $p$-value for comparison of adduct levels of responders versus nonresponders, Wilcoxon rank sum test.

plasmid remaining after DNAse treatment of cells at the end of the transfection procedure was about 3% of the amount of plasmid added to the transfection mixture. This is about the same amount of plasmid as was transfected into human ovarian cancer cells using DEAE dextran (*16*).

Although each of the lines was successfully transfected, only four (HuT 78, H9, MOLT-4, and WIL2-NS) successfully expressed chloramphenicol acetyltransferase activity at 24 or 48 hr after transfection. The other four cell lines studied have not been reported to have any DNA repair deficits, and cell survival studies suggest that sensitivity to cisplatin falls within the same broad range for

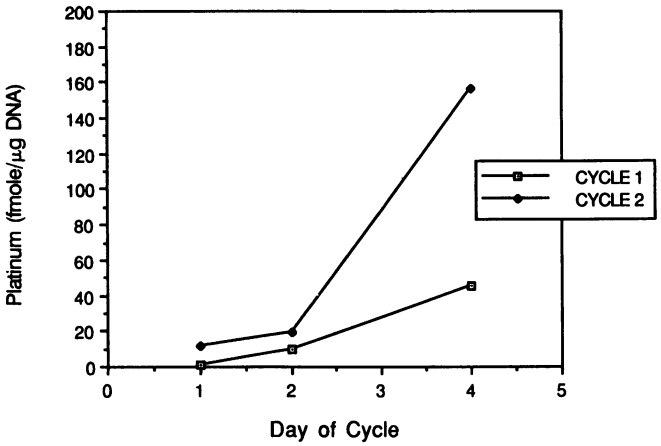


FIGURE 2. The mean adduct values are plotted for four patients who were studied on each of the 6 designated study days. Each data point represents the mean value on that day, calculated from data for these four individuals. (□) Data obtained from cycle 1 of therapy; (■) data obtained from cycle 2 of therapy.

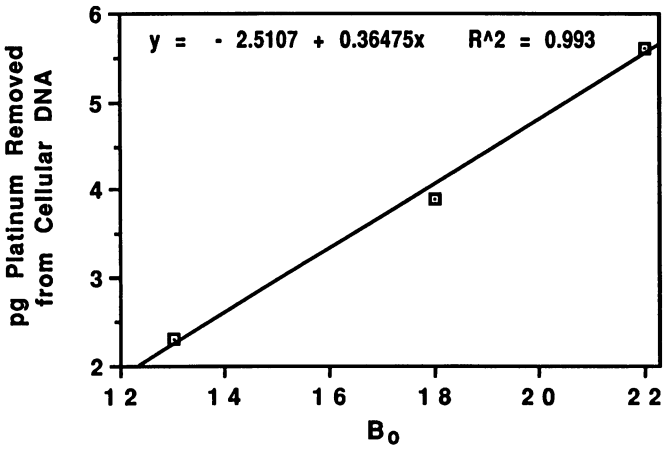


FIGURE 4. Two measures of DNA repair are related graphically for the three human T-lymphocyte cell lines. The horizontal axis represents the efficiency of cells to repair transfected cisplatin-modified plasmid DNA. The vertical axis represents the efficiency of cells to remove cisplatin from cellular DNA, as measured by atomic absorption spectrometry.

all eight cell lines (data not shown). These results indicate that cellular factors that allow repair and expression of transfected plasmid DNA may not be present in all peripheral blood leukocytes, even though there may be no apparent DNA repair deficit with respect to cellular DNA.

## Host-Cell Reactivation of Cisplatin-damaged Plasmid and Repair of Cellular DNA in T-Cells

Results obtained using our host-cell reactivation assay indicate that different subpopulations of blood cells have different DNA repair capabilities. We therefore took the approach that if we could study one cell type in each individual we could assess relative DNA repair capabilities between individuals for that cell type. Our first subpopulation of interest was T-lymphocytes: These cells are easily obtained, can be easily developed into a pure culture, and can be maintained without viral transformation for a period of months.

We measured the ability of the three T-cell lines to remove cisplatin from cellular DNA over 6 hr following exposure to 10 µM of the drug for 1 hr. HuT 78, H9, and MOLT-4 cells removed 5.6, 3.9, and 2.3 pg platinum per µg DNA, respectively. We next measured the ability of these cells to repair transfected cisplatin-damaged pRSVcat, using the host-cell reactivation assay. The $B_0$ for each of the cell lines was 22, 18, and 13, respectively. Plotting these two measures of DNA repair against one another gives the curve shown in Figure 4: the relationship is linear, with a correlation coefficient of 0.993.

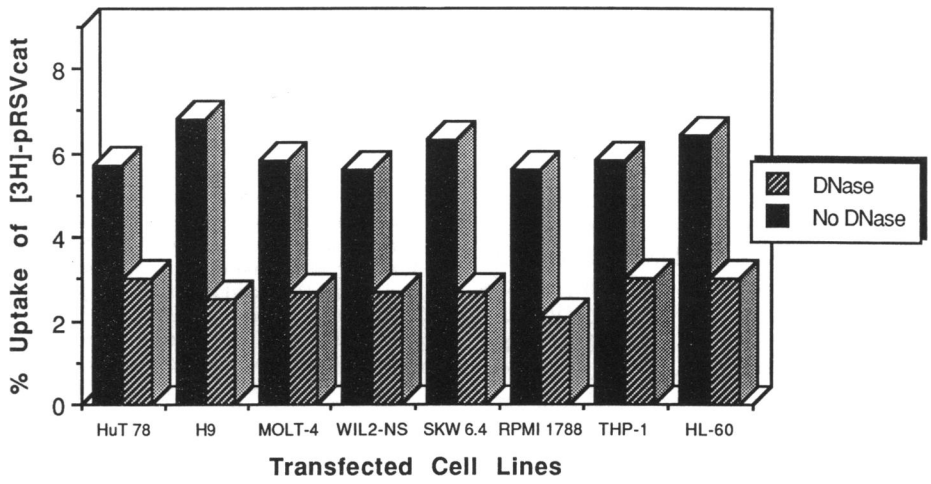


FIGURE 3. Efficiency of transfection of pRSVcat into each of eight human leukocyte cell lines, described in the Methods. Solid columns represent the percent uptake of $^3$H-labeled plasmid prior to DNAse treatment of cells (total cellular uptake of plasmid). Hatched columns represent the percent uptake of $^3$H-labeled plasmid after DNAse treatment of cells (presumed to represent intracellular plasmid).

## Differences in Host-Cell Reactivation between T- and B-Cells

It is currently unclear why only one of the three B-cell lines successfully expressed chloramphenicol acetyltransferase activity after transfection with cisplatin-modified plasmid. It is of note that the two B-cell lines that did not express this activity (SKW 6.4 and RPMI 1788) are both Epstein-Barr virus-transformed cell lines, whereas the WIL2-NS cell line is not. For the WIL2-NS cells, the $B_0$ is 8, which is lower than that of any of the T cell lines; and the $IC_{50}$ for cisplatin is 1.02 μM. Inclusion of the data for WIL2-NS in the data set for T-cells results in a breakdown of the mathematical relationship between the two assays we used to assess DNA repair. Thus, the relationship between DNA repair in cellular DNA and repair of transfected plasmid DNA holds only if the data set is restricted to one cell type: T-cells. Further, transformation of B cells by Epstein-Barr virus may be disadvantageous to the conduct of this assay.

# Discussion

In the clinical studies presented here, a cohort of 21 patients with 15 types of tumor received cisplatin and carboplatin as the only therapy for their malignancy. The relationship between platinum–DNA adduct level in peripheral blood cells and disease response was highly statistically significant, with a summary two-sided $p$-value of 0.00011. In studies of human ovarian cancer cells, DNA repair appears to be the most important single component of cellular resistance to cisplatin (*16*). In analyses of samples collected from patients at all the designated time points in this study, the absence of repair/removal of a portion of drug-induced DNA damage from peripheral blood cells appeared to contribute to an accumulation of adducts to higher than expected levels. Understanding the molecular processes that occur in patients will require direct study of cells from treated individuals. For that reason, we have developed a host-cell reactivation assay to assess cisplatin–DNA adduct repair capacity in human T-cells.

Human T-lymphocytes have been selected for study for several reasons. First, a close parallel between blood tissue and tumor tissue in the processing of platinum–DNA damage is strongly suggested by the data presented here and previous studies (*5–8*) and has been confirmed in studies of autopsied patients who received platinum-based therapy *ante mortem* (*10*). Second, different subpopulations of blood cells may be biologically different, so it is important to study the same cell type from all patients. T Cells are easily obtained and cultured, and DNA repair activity can be readily assessed by an assay that is amenable to the study of many samples—the host-cell reactivation assay.

In our studies, 30% of the adduct load seen in peripheral blood cells 24 hr after a cisplatin dose may persist for ≥ 25 days. The studies done by Fichtinger-Schepman and colleagues indicate that the adduct load at 24 hr may represent about one-quarter of the initial level of DNA damage (*21*). The persistent platinum–DNA adduct load may therefore represent 5–10% of the initial level of DNA damage induced by a cisplatin dose. Subsequent administration of carboplatin gives rise to the expected increase in DNA adduct level, whereas subsequent administration of cisplatin gives rise to a greater than expected increase in DNA adduct level (Fig. 2). One possible explanation for this finding may be that cisplatin covalently attacks DNA repair enzymes, in a fashion similar to its documented attack on kidney enzymes (*1,2*), and may thus partially inactivate DNA repair function.

Although many conclusions can be drawn from data generated by measuring DNA damage levels, a functional assessment of DNA repair capability would be important to test the validity of the hypotheses raised. Although measurement of levels of expression of human DNA repair genes and of the protein levels of relevant DNA repair enzymes would be important, a functional assessment of repair would overcome questions about artifactually elevated mRNA levels or high levels of enzyme that might be partially dysfunctional. For these reasons, the host-cell reactivation assay (which correlates well with cellular repair of cisplatin–DNA lesions in T-cells) shows promise as a means of addressing these questions.

Although many factors may contribute to resistance to cisplatin *in vitro* and in the clinic, an increasing body of evidence suggests that DNA repair is the single most important factor governing clinically relevant levels of resistance. The ability to measure platinum–DNA damage levels following therapeutic exposures has constituted the first major step in unraveling the questions of clinical resistance to platinum compounds. The next major step is to address the underlying molecular mechanisms which may modulate the level of DNA damage.

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