[CANCER RESEARCH 50, 1863–1866, March 15, 1990]

Increased Removal of DNA-bound Platinum in a Human Ovarian Cancer Cell Line Resistant to *cis*-Diamminedichloroplatinum(II)

Hidetaka Masuda,[1] Toshiko Tanaka, Hiroyuki Matsuda, and Izuo Kusaba

Department of Surgery, Kyushu Dental College, 2-6-1 Manazuru, Kokura-kita-ku, Kitakyushu 803, Japan

ABSTRACT

A human ovarian cancer cell line resistant to *cis*-diamminedichloroplatinum(II) (DDP) (2780^{CP}) was compared with its DDP-sensitive parental cell line (A2780) to determine whether differences in the removal rate of DNA-bound platinum were related to resistance. Both cell lines were treated *in vitro* with various doses of DDP for 2 h and subsequently incubated in arginine-deficient Eagle's minimum essential medium with 2.5% dialyzed fetal bovine serum in the presence or absence of aphidicolin. After 0, 12, and 24 h, DNA was isolated from the cells and DNA-bound platinum was determined by flameless atomic absorption spectrophotometry.

Binding of platinum to DNA of either cell line was a linear function of concentration ranging from 20 to 80 μM DDP. Platinum binding was almost equal at each dose in both cell lines. 2780^{CP} cells that were 3-fold resistant to DDP lost 30.5 to 40.1% of their total DNA-bound platinum, compared with a 1.3 to 16.1% loss for A2780 cells, 12 to 24 h after a 2-h exposure to 40 μM DDP, respectively.

Aphidicolin (3.0 μg/ml) increased the cytotoxicity in 2780^{CP} cells by about 2-fold and caused a significant delay in the time required for platinum removal in the resistant cells (14.6 and 18.9% at 12 and 24 h).

These studies indicate that the mechanism of DDP resistance in the 2780^{CP} cell line is related to an increased ability to remove platinum-DNA adducts, and not to a difference in initial DDP binding to DNA.

INTRODUCTION

DDP[2] is an important antitumor agent proved effective against a broad range of human malignancies (1–3). However, acquired resistance to DDP strongly limits its clinical use (4, 5). Several mechanisms of DDP resistance in human and rodent cell lines have been described (6–15). Recently, an increased ability of a DDP-resistant human ovarian cancer cell line, 2780^{CP}, to repair DDP-induced DNA damage has been proposed as a mechanism of DDP resistance. This suggestion was based on experiments using the technique of unscheduled DNA synthesis or alkaline CsCl equilibrium sedimentation (16–19). These findings prompted us to examine whether the 2780^{CP} cell line actually removed platinum lesions from their DNA more rapidly than the sensitive parental cell line (A2780) since the sensitivity of cells to DDP is directly related to the amount of platinum remaining in the DNA (20, 21). In this study, we found that platinum loss from DNA, as measured by atomic absorption spectrophotometry, during the initial 24 h after DDP treatment reflected DNA repair activity and was correlated with DDP resistance in the 2780^{CP} cell line.

MATERIALS AND METHODS

Chemicals. MEM, ADM, and kanamycin were obtained from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. FBS, DFBS, and trypsin were from GIBCO, Grand Island, NY. DDP was provided by Nippon Kayaku Co., Tokyo, Japan. SDS, glutamine, diphenylamine, trichloroacetic acid, aphidicolin, and platinum standard solution (H_2PtCl_6 in 1 N HCl) were purchased from Wako Pure Chemical Co., Ltd., Tokyo, Japan. DNA, RNase A, and RNase T_1 were from Sigma Chemical Co., St. Louis, MO. Proteinase K was obtained from Merck, Darmstadt, West Germany.

Cell Lines and Culture. A2780, an ovarian cancer cell line derived from an untreated patient, and 2780^{CP}, a DDP-resistant cell line, were the kind gifts of Drs. R. F. Ozols and T. C. Hamilton, Fox Chase Cancer Center, Philadelphia, PA. The 2780^{CP} cell line was produced by exposure of the A2780 cell line to a stepwise-increasing concentration of the drug up to 70 μM. Both cell lines were maintained as monolayers in MEM supplemented with 10% (v/v) FBS, 60 μg/ml kanamycin, and 0.3 mg/ml glutamine at 37°C in a humidified 5% CO_2-95% air atmosphere.

Drug Treatment. Cell lines were plated in culture dishes at high density (8×10^5 cells/21-cm^2-dish for the cytotoxicity studies, 5×10^6 cells/145-cm^2 dish for the Pt-DNA removal assays) in MEM with 10% FBS and grown to confluence for 72 h. At this point, cultures were switched to ADM with 2.5% DFBS to inhibit further replication of DNA (22, 23). After 24 h (day 4 in culture), the cells were treated with various doses of DDP for 2 h, washed 3 times with phosphate-buffered saline, and incubated in fresh ADM containing 2.5% DFBS for the required time intervals at 37°C with or without aphidicolin.

Cytotoxicity Studies. A stationary phase culture in a 21-cm^2 dish was used to test DDP sensitivity under the same conditions as the repair experiment. This is a cell-killing assay, not a cell growth inhibition assay. After treatment with DDP, the cells were incubated for 72 h in ADM with 2.5% DFBS, washed 3 times, trypsinized, and counted in a Coulter Counter (Coulter Electronics, Ltd., Luton, England).

DNA Isolation from the Cells. Cells that were treated with DDP and subsequently incubated in ADM containing 2.5% DFBS for the required time interval were collected by centrifugation at 1000 rpm after 10 min. Two dishes (145 cm^2) were used for each data point. The pellets were washed once with phosphate-buffered saline and lysed by 2.5 ml of 10 mM Tris-HCl/400 mM NaCl/10 mM EDTA/0.5% (w/v) SDS, pH 8. Proteinase K (200 μg/ml) was added and incubated for 3 h at 37°C to digest the protein in the lysate. The solutions were extracted twice with an equal volume of phenol: $CHCl_3$:isoamyl alcohol (50:48:2) saturated with 100 mM Tris-HCl/10 mM EDTA/10 mM NaCl/0.5% SDS (w/v), pH 8.15, and DNA was precipitated from the aqueous phase with 2 volumes of cold absolute ethanol after standing overnight at −20°C. The DNA was spooled on glass rods, rinsed in 80% ethanol, and dissolved in 1 ml of 10 mM Tris-HCl/10 mM NaCl/10 mM EDTA, pH 8. Coprecipitated RNA was subsequently digested in a 2-h incubation at 37°C with 40 μg/ml of RNase A and 10 μg/ml of RNase T_1. This was followed by a phenol extraction and an alcohol precipitation as above. After being spooled, rinsed, and dried, the DNA was dissolved in 5% trichloroacetic acid (200 μl) at 80°C for 20 min. A portion of the DNA solution was used to measure platinum content, and the remaining portion was used to quantify DNA content via the Burton's diphenylamine procedure (24).

Platinum Determinations. Pt-DNA was determined by injecting a 10-μl sample into a pyrocoated graphite cuvette (Hitachi Model 180-7444) using a Hitachi polarized Zeeman Model z-8100 flameless atomic absorption spectrophotometry (Hitachi, Ltd., Tokyo, Japan). For calibration, a standard platinum solution was used. The platinum content was expressed as pg/μg DNA.

RESULTS AND DISCUSSION

It is especially important to determine that both A2780 and 2780^{CP} cells are in the stationary phase during the Pt-DNA

Received 6/21/89; revised 12/1/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: DDP, *cis*-diamminedichloroplatinum(II); Pt-DNA, DNA-bound platinum; FBS, fetal bovine serum; DFBS, dialyzed FBS; MEM, Eagle's minimum essential medium; ADM, arginine-deficient MEM; SDS, sodium dodecyl sulfate; IC_{50}, dose level resulting in 50% inhibition; GSH, glutathione; dNTP, deoxynucleotide triphosphate.

removal assay since proliferating cells would excise platinum-induced lesions faster than nonproliferating cells (21). As shown in Fig. 1, the number of A2780 and 2780^{CP} cells was unchanged from day 4 (24 h after the first replacement of medium by ADM) through day 6 despite a second replacement of ADM on day 4 for posttreatment incubation. The coefficients of variation in cell number were 3.34 and 3.64% for A2780 and 2780^{CP} cells, respectively. A Pt-DNA removal assay, therefore, was done on days 4 to 5.

Fig. 2 shows the survival rates of A2780 and 2780^{CP} cells in the presence or absence of aphidicolin after a 2-h treatment with increasing concentrations of DDP. By comparing IC_{50} drug doses for A2780 and 2780^{CP} cells, 2780^{CP} cells were found to be 3-fold more resistant to DDP under the conditions used (72 μM IC_{50} 2780^{CP}, 24 μM IC_{50} A2780). Our preliminary experiment showed that aphidicolin alone had no effect on the survival of either cell line at doses below 5 μg/ml. As shown in Fig. 2, 3 μg aphidicolin/ml increased DDP cytotoxicity in the 2780^{CP} line by about 2-fold (72 μM IC_{50} 2780^{CP}, 44 μM IC_{50} 2780^{CP} with aphidicolin).

Several researchers have reported that a decrease in DDP accumulation may be an important means of DDP resistance (8–10). Accordingly, any difference in the amount of platinum binding to DNA between A2780 and 2780^{CP} cell lines should be assessed. As shown in Fig. 3, the platinum bound to DNA of either cell line increased linearly with doses up to 80 μM. It was noteworthy that the amount of Pt-DNA was nearly equal in both cell types at each DDP dose tested. This result is essential for comparing the repair ability of each cell line on the basis of equimolar binding of DDP to cellular DNA.

Fig. 4 shows the removal of Pt-DNA in A2780 and 2780^{CP} cells and the effect of aphidicolin on Pt-DNA removal in 2780^{CP} cells. At 12 and 24 h after a 2-h exposure to 40 μM DDP, A2780 cells lost only 1.3 and 16.1% of their total Pt-DNA, compared with 30.5 and 40.1% for 2780^{CP} cells. Thus, 2780^{CP} cells had a significantly higher rate of platinum removal than A2780 cells ($P < 0.05$, 12 and 24 h). These results include a comparison of the removal rates at equimolar binding of platinum to DNA in both cell lines as obtained from the data shown in Fig. 3. Furthermore, for equitoxic comparison of the removal rates, 2780^{CP} cells were exposed to 80 μM DDP (60% cell killing doses are 40 μM for A2780 cells and 80 μM for 2780^{CP} cells; see Fig. 2). The removal of platinum in 2780^{CP} cells was more rapid by 36.9% at 12 h and 51.9% at 24 h after DDP treatment ($P < 0.02$, 12 h; $P < 0.05$, 24 h). Meanwhile, in the presence of 3 μg aphidicolin/ml, the platinum removal rates in 2780^{CP} cells were 14.6% at 12 h and 18.9% at 24 h after drug treatment. Thus, aphidicolin caused a significant delay in plat-

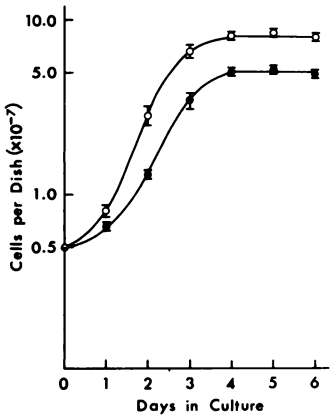

Fig. 1. Growth of A2780 (○) and 2780^{CP} (●) after replacement of medium with ADM plus 2.5% DFBS. Cell lines were plated in 145-cm^2 dishes at high density (5×10^6) in MEM with 10% FBS and grown to confluence for 72 h. At this point (day 3), cultures were switched to ADM containing 2.5% DFBS. After 24 h (day 4), culture medium was replaced a second time by fresh ADM plus 2.5% DFBS. Cells were counted daily. *Bars*, SD in 3 determinations.

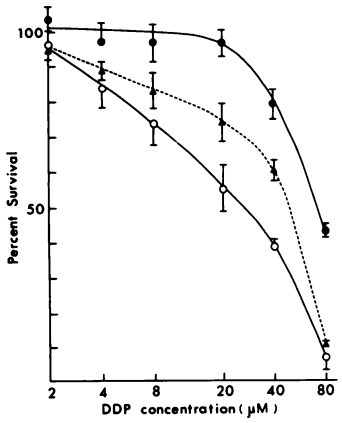

Fig. 2. Survival of A2780 (○) and 2780^{CP} (●), and the effect of aphidicolin on survival of 2780^{CP} (▲) as a function of DDP concentration. Cells were incubated for 72 h at 37°C in ADM plus 2.5% DFBS with or without 3 μg aphidicolin/ml after DDP treatment for 2 h and then counted. Results were expressed as a percentage of control. *Bars*, SD in 3 determinations.

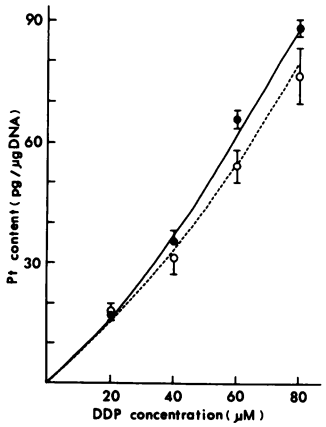

Fig. 3. Dose-dependent increase of platinum bound to DNA to A2780 (○) and 2780^{CP} (●). Stationary phase cultures were treated with various concentrations of DDP for 2 h at 37°C. Immediately after drug removal, DNA was extracted and purified, and Pt-DNA was measured. *Bars*, SD in 3 determinations.

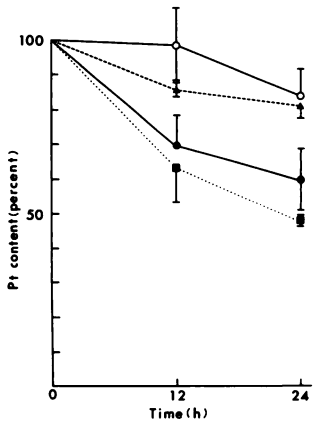

Fig. 4. Kinetics of the removal of Pt-DNA and effect of aphidicolin. Stationary phase cultures were exposed to DDP [40 μM, A2780 (○), 40 μM, 2780^{CP} (●) and 80 μM, 2780^{CP} (■)] for 2 h at 37°C. The amounts of Pt-DNA remaining after a 12- and 24-h incubation period in ADM plus 2.5% DFBS in the absence or presence [40 μM DDP, 2780^{CP} (▲)] of aphidicolin (3 μg/ml) were plotted relative to the amount initially bound ($t = 0$; 100%). Zero time is immediately after drug removal. *Bars*, SD in 3 determinations.

inum removal in the resistant cells ($P < 0.05$, 12 and 24 h). There was no significant difference in the platinum removal rates between 2780^{CP} cells with aphidicolin and A2780 cells.

In this time course study, we confirmed previous reports (16–19) concerning the relevance of DNA repair ability on DDP resistance. We measured the amount of Pt-DNA in nondividing cells using ADM and DFBS to separate excision repair from postreplication repair (21–23, 25). This method is simpler and more reliable than a sedimentation analysis as far as its ability to excise platinum-DNA adducts is concerned since sedimentation analysis reflects a resynthesis step of excision repair.

It is worth noting that the amount of platinum bound to the DNA of A2780 and 2780^{CP} cells was almost the same for each DDP dose tested (Fig. 3). These results are inconsistent with reduced DNA cross-linking as a potential mechanism of resistance, which would lead to decreased platinum-DNA binding (6, 7). However, the Pt-DNA we measured included both platinum-DNA monoadducts and platinum-DNA cross-links, and the conversion of monoadducts to cross-links may be low in the resistant cells. It is not known which type of platinum-DNA adducts are removed more rapidly in the resistant cells. Recently, Hospers *et al.* (7) reported a decreased Pt-DNA binding after a 1-h treatment at DDP doses over 100 μM in a human small cell lung carcinoma cell line resistant to DDP. Our data were not directly comparable because we did not use such high concentrations of DDP. This was due to high cytotoxicity in both cell lines. Furthermore, each cell line may have its own resistant mechanism.

Because alterations in the plasma membrane can have an effect on Pt-DNA binding, a comparison of the repair ability of each cell type on the basis of equimolar binding of the drug to cellular DNA is essential. In the present study, this can be seen by comparing equimolar concentrations in the medium (Figs. 3 and 4).

Cell viability may also have an effect on repair ability. Therefore, we compared the removal rate of Pt-DNA among the respective cell lines at the equitoxic DDP level. A higher Pt-DNA removal rate was demonstrated in the DDP-resistant 2780^{CP} cells as compared to the parental A2780 cells (Fig. 4), confirming previous reports (18).

Lai *et al.* (19) showed that in 2780^{CP} cells GSH reduction mediated by buthionine sulfoximine led to a decrease in DNA repair activity after DDP exposure, probably due to the reduced pool size of DNA precursors, dNTPs through GSH reduction. However, 2780^{CP} cells have a higher GSH content than A2780 cells (11, 16). These observations might suggest that an increased GSH content in 2780^{CP} cells causes an increase in their repair activity through larger dNTP pools, compared to A2780 cells. Lu *et al.* (26) reported an increased dTMP synthase activity in DDP-resistant A2780 cells, which would lead to an increase in a DNA precursor, dTMP. However, it is not known whether this result is related to increased DNA repair.

Aphidicolin, a specific inhibitor of DNA polymerase α (27, 28), inhibited, in part, excision of Pt-DNA and increased the DDP cytotoxicity (Figs. 2 and 4). These findings are also consistent with previous reports (17, 19) and suggest that DNA polymerase α would be partially involved in the repair of 2780^{CP} cells after DDP-induced DNA damage although the efficiency of repair inhibition of aphidicolin may be dictated by dNTP pool (29). We are now examining changes of overall activity of this enzyme in the resistant cells. In conclusion, the DDP-resistant phenotype of a human ovarian cancer cell line, 2780^{CP}, is associated with an increased ability to repair DDP-induced DNA damage.

ACKNOWLEDGMENTS

We thank Drs. R. F. Ozols and T. C. Hamilton for kindly providing A2780 and 2780^{CP} cells. We also thank Dr. Terrell E. Jones for his critical reading of the manuscript.

REFERENCES

1. Prestayko, A. W., Crooke, S. T., and Carter, S. K. Cisplatin: Current Status and New Developments. New York: Academic Press, 1980.
2. Loehrer, P. J., and Einhorn, L. H. Cisplatin. Ann. Intern. Med., *100:* 704–713, 1984.
3. Rosenberg, B. Fundamental studies with cisplatin. Cancer (Phila.), *55:* 2303–2316, 1985.
4. Ozols, R. F., and Young, R. C. Chemotherapy of ovarian cancer. Semin. Oncol., *11:* 251–263, 1984.
5. Ozols, R. F. Pharmacologic reversal of drug resistance in ovarian cancer. Semin. Oncol., *12* (Suppl. 4): 7–11, 1985.
6. Zwelling, L. A., Michaels, S., Schwartz, H., Dobson, P. P., and Kohn, K. W. DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukemia to *cis*-diamminedichloroplatinum(II) and L-phenylalanine mustard. Cancer Res., *41:* 640–649, 1981.
7. Hospers, G. A. P., Mulder, N. H., de Jong, B., de Ley, L., Uges, D. R. A., Fichtinger-Schepman, A. M. J., Scheper, R. J., and de Vries, E. G. E. Characterization of a human small cell lung carcinoma cell line with acquired resistance to *cis*-diamminedichloroplatinum(II) *in vitro*. Cancer Res., *48:* 6803–6807, 1988.
8. Kuppen, P. J. K., Schuitemaker, H., van't Veer, L. J., de Bruijin, E. A., van Oosterom, A. T., and Schrier, P. I. *cis*-Diamminedichloroplatinum(II)-resistant sublines derived from two human ovarian tumor cell lines. Cancer Res., *48:* 3355–3359, 1988.
9. Kraker, A. J., and Moor, C. W. Accumulation of *cis*-diamminedichloroplatinum(II) and platinum analogues by platinum-resistant murine leukemia cells *in vitro*. Cancer Res., *48:* 9–13, 1988.
10. Andrews, P. A., Velury, S., Mann, S. C., and Howell, S. B. *cis*-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Res., *48:* 68–73, 1988.
11. Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Behrens, B. C., Tsuruo, T., Grotzinger, K. R., Mckoy, W. M., Young, R. C., and Ozols, R. F. Augmentation of Adriamycin, melphalan and cisplatin cytotoxicity in drug resistant and sensitive human ovarian cancer cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem. Pharmacol., *34:* 2583–2586, 1985.
12. Hromas, R. A., Andrews, P. A., Murphy, M. P., and Burns, C. P. Glutathione depletion reverses cisplatin resistance in murine L1210 leukemia cells. Cancer Lett., *34:* 9–13, 1987.
13. Bakka, A., Endresen, L., Johnsen, A. B. S., Edminson, P. D., and Rugstad, H. E. Resistance against *cis*-dichlorodiammineplatinum(II) in cultured cells with a high content of metallothionein. Toxicol. Appl. Pharmacol., *61:* 215–226, 1981.
14. Andrews, P. A., Murphy, M. P., and Howell, S. B. Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. Cancer Chemother. Pharmacol., *19:* 149–154, 1987.
15. Richon, V. M., Schulte, N., and Eastman, A. Multiple mechanisms of resistance to *cis*-diamminedichloroplatinum(II) in murine leukemia L1210 cells. Cancer Res., *47:* 2056–2061, 1987.
16. Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., Mckoy, W. M., Young, R. C., and Ozols, R. F. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res., *47:* 414–418, 1987.
17. Masuda, H., Ozols, R. F., Lai, G. M., Fojo, A., Rothenberg, M., and Hamilton, T. C. Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. Cancer Res., *48:* 5713–5716, 1988.
18. Lai, G. M., Ozols, R. F., Smyth, J. F., Young, R. C., and Hamilton, T. C. Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. Biochem. Pharmacol., *37:* 4597–4600, 1988.
19. Lai, G. M., Ozols, R. F., Young, R. C., and Hamilton, T. C. Effect of glutathione on DNA repair in cisplatin-resistant human ovarian cancer cell lines. J. Natl. Cancer Inst., *81:* 535–539, 1989.
20. Fraval, H. N. A., and Roberts, J. J. Effects of cisplatin(II)diammine-dichloride on survival and the rate of DNA synthesis in synchronously growing Chinese hamster V79.379A cells in the absence and presence of caffeine. Evidence for an inducible repair mechanism. Chem.-Biol. Interact., *23:* 99–110, 1978.
21. Fraval, H. N. A., and Roberts, J. J. Excision repair of *cis*-diamminedichloroplatinum(II)-induced damage to DNA of Chinese hamster cells. Cancer Res., *39:* 1793–1797, 1979.
22. Stich, H. F., and San, R. H. C. DNA repair and chromatid anomalies in mammalian cells exposed to 4-nitroquinoline 1-oxide. Mutat. Res., *10:* 389–404, 1970.
23. Trosko, J. E., and Yager, J. D. A sensitive method to measure physical and chemical carcinogen-induced unscheduled DNA synthesis in rapidly dividing eukaryotic cells. Exp. Cell Res., *88:* 47–55, 1974.
24. Burton, K. A study of the conditions and mechanism of the diphenylamine

reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., *62:* 315–323, 1956.
25. Cleaver, J. E. Methods for studying repair of DNA damaged by physical and chemical carcinogens. Methods Cancer Res., *11:* 123–165, 1975.
26. Lu, Y., Han, J., and Scanlon, K. J. Biochemical and molecular properties of cisplatin-resistant A2780 cells grown in folinic acid. J. Biol. Chem., *263:* 4891–4894, 1988.
27. Dresler, S. L., and Lieberman, M. W. Identification of DNA polymerases involved in DNA excision repair in diploid human fibroblasts. J. Biol. Chem., *258:* 9990–9994, 1983.
28. Yamada, K., Hanaoka, F., and Yamada, M. Effects of aphidicolin and/or 2′,3′-dideoxythymidine on DNA repair induced in HeLa cells by four types of DNA-damaging agents. J. Biol. Chem., *260:* 10412–10417, 1985.
29. Snyder, R. D., and Regan, J. D. Differential responses of log and stationary phase human fibroblasts to inhibition of DNA repair by aphidicolin. Biochim. Biophys. Acta, *697:* 229–234, 1982.

Increased Removal of DNA-bound Platinum in a Human Ovarian Cancer Cell Line Resistant to *cis*-Diamminedichloroplatinum(II)

Hidetaka Masuda, Toshiko Tanaka, Hiroyuki Matsuda, et al.

Cancer Res 1990;50:1863-1866.