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Flow Cytometric Analyses of the Characteristics of Tumor Cells Treated with Two Platinum Compounds: 1,1-Cyclobutanedicarboxylato(2-aminomethylpyrrolidine)-platinum(II) and Cisplatin

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In order to reduce the toxicities of cisplatin (DDP) and/or to improve antitumor efficacy, a large number of new platinum analogues have been synthesized. 1,1-Cyclobutanedicarboxylato(2-aminomethylpyrrolidine)platinum(II) (DWA2114R) is one of them. In this study, we characterized the action mechanism of DWA2114R flow-cytometrically in 3 human lung cancer cell lines by using bromodeoxyuridine (BrdUrd), rhodamine 123 (Rho) and Ki-67 antibody (Ab), and compared the results with those for DDP. We found that the actions of these 2 platinum analogues were characteristically different at the subcellular level. Our observations may be summarized as follows. a) Simultaneous exposure of cells to DDP and BrdUrd resulted in decreases in fluorescence intensity, i.e. in the amount of BrdUrd incorporated into single-stranded DNA. b) DDP appears to be approximately 20-fold more active than DWA2114R in producing cell cycle perturbation. c) In PC-6 small cell carcinoma cells, DDP induced decreases in S phase cells and accumulation of cells in the G_2M phase, whereas in PC-10 squamous carcinoma and PC-3 adenocarcinoma cells DDP produced S phase cell accumulation. Weak but similar changes occurred with DWA2114R. d) The high Ki-67 antigen cell population was decreased by treatment with either DDP or DWA2114R, but DDP reduced the low Ki-67 antigen population more than DWA2114R. e) In PC-10 and PC-6 cells, DDP suppressed Rho incorporation into live mitochondria, whereas DWA2114R produced no change in Rho incorporation. PC-3 cells were not affected by either DDP or DWA2114R. It is likely that these differences reflect the biological activities of DDP and DWA2114R.

Key words: Platinum compounds — Flow cytometry — Bromodeoxyuridine — Ki-67 antigen — Rhodamine 123

Cisplatin (DDP) is one of the most active anticancer agents currently in clinical use. Its side effects are considerable, however, and include severe nausea/vomiting, neurotoxicity, and dose-limiting nephropathy.[1,2] In order to reduce these toxicities, and/or to improve antitumor efficacy, a large number of platinum analogues have been synthesized. 1,1-Cyclobutanedicarboxylato(2-aminomethylpyrrolidine)platinum(II) (DWA2114R), recently developed in Japan, is one of them. Animal studies showed that DWA2114R was as active as cisplatin against P388 and L1210 leukemias, and Lewis lung, Meth A, C3MC2, M5076, Colon 26, Colon 38 and Walker 256 carcinomas, but was devoid of nephrotoxicity.[3,4] Phase III studies of DWA2114R are in progress in Japan.

In the present study, we characterized the action of DWA2114R flow-cytometrically in three human lung cancer cell lines by using bromodeoxyuridine (BrdUrd), rhodamine 123 (Rho) and Ki-67 antibody (Ab), and compared the results with those for DDP. BrdUrd is known to be incorporated into single-stranded DNA in S phase,[5,6] Rho into live mitochondria[7,8] and Ki-67 Ab into proliferating cells.[9,10] We found that these 2 platinum analogues displayed different characteristic effects at the subcellular level.

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MATERIALS AND METHODS

Cells Three human lung cancer cell lines, PC-3 adenocarcinoma, PC-10 squamous cell carcinoma and PC-6 small cell carcinoma, were used.[11] These cell lines were maintained as monolayers in RPMI-1640 medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO) ("complete medium") in a humidified 5% CO_2/air atmosphere. These cells were subcultured weekly after trypsinization with a solution containing 0.1% trypsin (Fraction 3, Sigma, St. Louis, MO) and 0.02% EDTA (Sigma). The population doubling times of PC-3, PC-10 and PC-6 were 27, 23 and 20 h, respectively.

Cell growth inhibition assay DDP was obtained from Nippon Kayaku Co., Tokyo, and DWA2114R from

Chugai Pharmaceutical Co., Tokyo. Cells in monolayer were trypsinized and the viable cell density was adjusted to 1×10^5 cells/ml by the trypan blue dye exclusion method. Aliquots of 5 ml of cells were seeded in 60 mm Petri dishes and preincubated for 24 h in order for cells to attach to the floor of the dishes and to enter the logarithmic growth phase. After confirmation that the cells had become attached to the dish as a monolayer on the following day, they were exposed to graded concentrations of DDP or DWA2114R for 20 min or 1 h, washed thrice with Dulbecco's phosphate-buffered saline (PBS) and recultured in the complete medium for 24 h. Cells were trypsinized again and viable cells were enumerated by using the trypan blue dye exclusion method. Because we wished to assess the mechanisms of action of platinum compounds, a 24 h incubation period after drug exposure was selected, as it corresponds roughly to a population doubling time.

Monoclonal antibodies and dyes for flow cytometry BrdUrd was obtained from Becton Dickinson, San Jose, CA, anti-BrdUrd mouse Ab and Ki-67 Ab from Dakopatts, Denmark; and Rho and propidium iodide (PI) from Sigma. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG goat Ab as the second Ab was also obtained from Dakopatts.

Drug exposure and staining methods In this study BrdUrd was used for two purposes: to evaluate interactions of BrdUrd and platinum compounds with DNA, and to observe perturbing effects of the compounds on the cell cycle. Cells were exposed to BrdUrd and platinum compounds either simultaneously or sequentially. For the simultaneous exposure, cells were exposed to BrdUrd and DDP or DWA2114R for 20 min. Additionally, in order to evaluate cell cycle-perturbing effects, cells were exposed to DDP or DWA2114R for 20 min without BrdUrd followed by the procedures described below.

For sequential exposure, cells were exposed to drug for 1 h, washed free of drug and recultured. BrdUrd was added 20 min before the end of the 24 h culture period. Cells were then washed with cold PBS thrice, trypsinized and fixed overnight with ethanol at 4 °C. For the staining of BrdUrd with FITC-conjugated anti-BrdUrd Ab, the method of Schutte *et al.*[12] was modified as follows. Fixed cells were washed twice with PBS and nuclei were isolated by exposure to pepsin (Sigma, final concentration 0.2 mg/ml in 0.1 *N* HCl) at 37°C for 5 min. Pepsin was inactivated by adding 0.05% Tween 20 PBT buffer (PBS containing 100 mg/dl bovine serum albumin (BSA, Sigma) and 0.05% Tween 20, pH 7.4). Isolated nuclei were washed once with PBS and then incubated with 2 *N* HCl for 30 min to denature DNA. Immediately thereafter, the solution was neutralized with borax buffer (0.1 *M* sodium tetraborate, pH 8.5), washed twice with 0.05% Tween 20 PBT buffer and incubated with anti-BrdUrd mouse antibody (1:200 diluted) at 4°C. After overnight incubation, nuclei were washed twice with 0.05% Tween 20 PBT and exposed to rabbit anti-mouse Ig-G-FITC (1:50 diluted) for 1 h at room temperature. The samples were then stained with PI (50 μg/ml PI and 1.12% sodium citrate solution) and subjected to flow-cytometric analysis.

For the Ki-67 antigen (Ag) staining, the method of Schwarting *et al.*[10] was modified as follows. After reculture following drug exposure, cells were trypsinized, washed once with cold PBS and fixed with acetone (stored at −70°C) for 5 min. The samples were then washed with cold PBS and cold PBT (100 mg/dl BSA and 0.5% Tween 20). Fixed cells were incubated with anti-Ki-67 mouse Ab (1:50 diluted) for 1 h at 4°C. After washing with cold PBS and PBT, cells were resuspended in rabbit anti-mouse IgG-FITC (1:50 diluted) for 1 h at 4°C. The samples were then stained with PI and subjected to analysis.

Live mitochondrial staining with Rho was carried out as described by Darzynkiewicz *et al.*[7] Briefly, 24 h after drug exposure, Rho was added to the medium (final concentration 10 μg/ml) and cells in monolayer were exposed to Rho for 30 min at 37°C. Cells were then washed with PBS and trypsinized. Cell suspensions were washed twice with cold PBS and kept in PBS at 0°C until measurement.

Flow cytometric analysis Cell samples were analyzed using a system 50H Cytofluorograf (Ortho Diagnostics, Westwood, MA) linked to a digital computer Model 2150 (Ortho Diagnostics). The 488 nm line of a 300 mW argon ion laser was used for excitation. The two-color fluorescence emission was measured at 530 nm using a band-pass filter and beyond 630 nm using a long-pass filter. The flow cytometer was standardized using 2.00 μm Lactec particles (Poly-science, Inc., Warrington, PA).

RESULTS

Effects of DDP and DWA2114R on cell growth The effects of DDP or DWA2114R on cell growth are shown in Fig. 1. Among the cell lines tested, PC-6 cells were the most sensitive to both DDP and DWA2114R, There was a progressive decrease in cell growth with increasing DDP concentration; however, the dose-response curves of PC-10 and PC-3 cell lines reached a plateau at high drug concentrations. This finding suggests the existence of a subpopulation of cells resistant to DDP. When cells were treated with DWA2114R, growth decreased progressively with increasing drug concentration. The slope of PC-6 was the steepest and was similar to that in the case of DDP treatment. Although DWA2114R was less

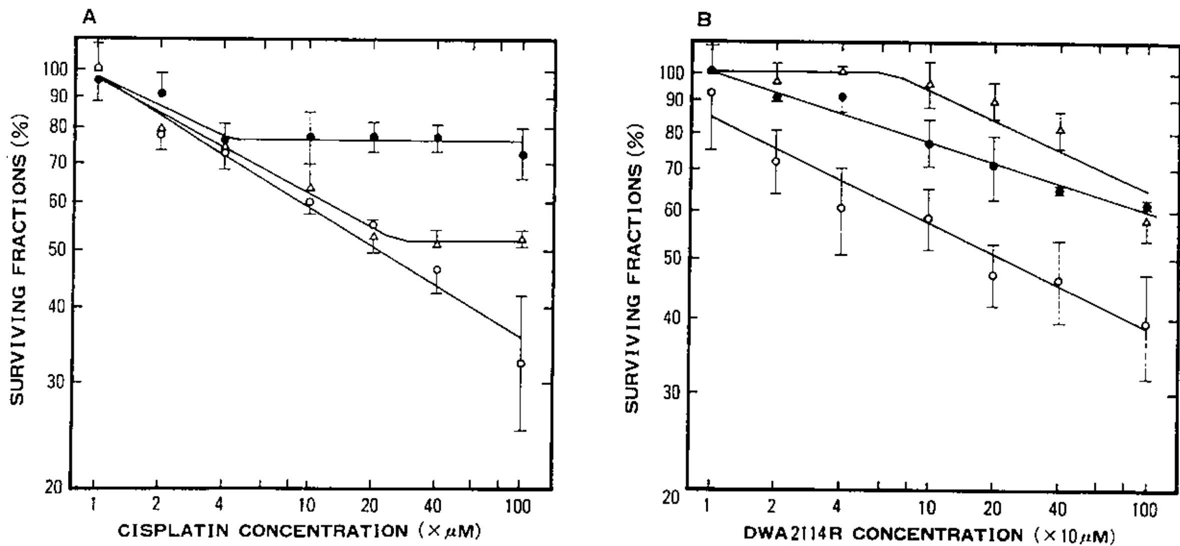
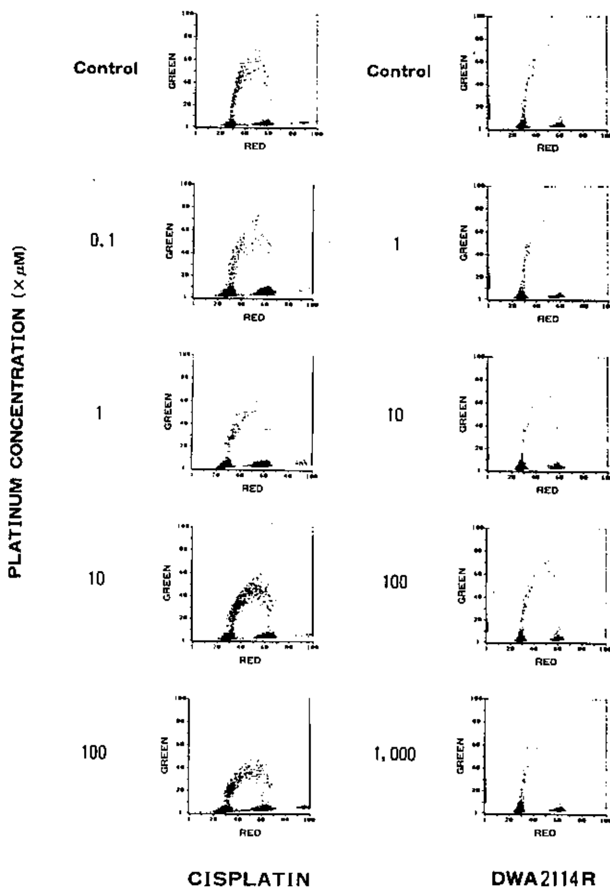


Fig. 1. Inhibition of growth of PC-10 (△), PC-3 (●), and PC-6 cells (○) treated with cisplatin (Panel A) and DWA2114R (Panel B) for 1 h. Viable cells were counted by the trypan blue dye exclusion method 24 h after the exposure. The data points are the combined averages of three experiments and error bars indicate SD.



active against PC-10 and PC-3 cells than PC-6 cells, the dose-response curves were different from those of DDP.

Interactions of DDP and DWA2114R with BrdUrd Simultaneous exposure of PC-10 cells to DDP at a concentration of more than 1 μM with BrdUrd for 20 min resulted in reduced BrdUrd fluorescence levels (Fig. 2). DWA2114R caused no suppression of BrdUrd incorporation even at a drug concentration as high as 1,000 μM. Results of sequential exposure to DDP or DWA2114R followed by BrdUrd are shown in Fig. 3. Cell cycle perturbation was observed when PC-10 cells were treated with DDP only at the concentrations (not lower than 10 μM) that reduced BrdUrd fluorescence. Exposure to DWA2114R for 20 min gave rise to no cell cycle perturbation, even at 1,000 μM.

Comparison of cell cycle perturbation caused by DWA-2114R and by DDP In the case of PC-6 cells treated with increasing concentrations of DDP and DWA2114R, the

Fig. 2. Influence of simultaneous exposure of cells to a platinum compound and BrdUrd on BrdUrd incorporation into DNA. PC-10 cells were simultaneously exposed to graded concentrations of cisplatin (left panel) or DWA2114R (right panel) and 10 μM BrdUrd for 20 min. The vertical axes indicate levels of BrdUrd incorporated into DNA. Mean channel numbers in the mid-S phases are 56.5 ± 15.1,* 52.5 ± 17.1, 49.2 ± 15.9, 41.3 ± 9.8, and 34.8 ± 10.1 from top to bottom of the left panel, whereas corresponding values range between 54.1 and 63.5 in the right panel. The data refer to the mean±SD. * $P\leq0.01$ versus the bottom three cytograms of the left panel.

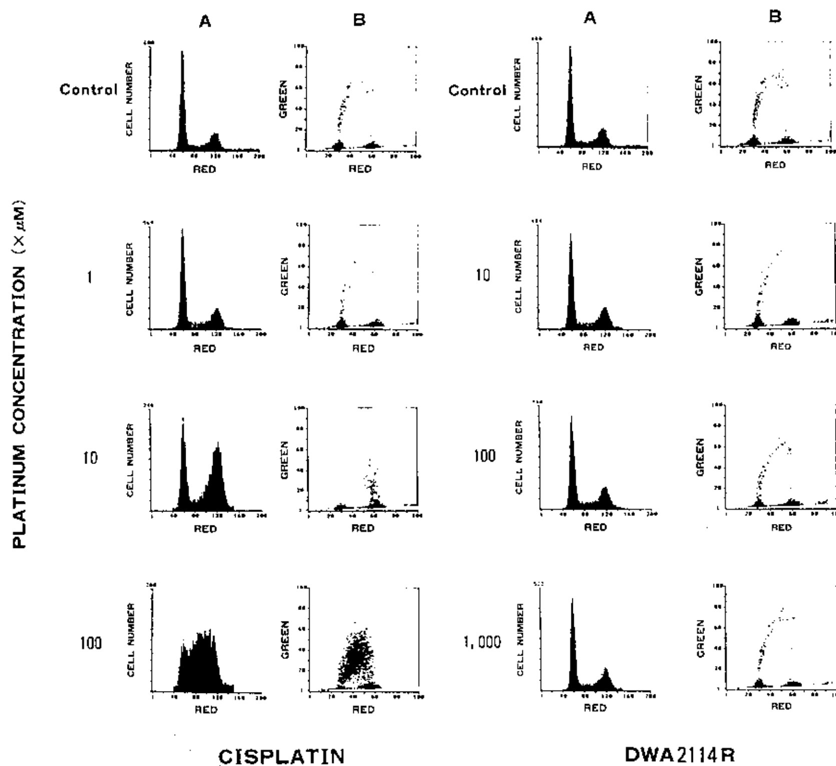


Fig. 3. DNA histograms (panel A) and bivariate BrdUrd (GREEN)/DNA (RED) cytograms (panel B) of PC-10 cells exposed to cisplatin or DWA2114R for 20 min. BrdUrd was added 20 min before the end of 24 h reculture.

percentage of cells in the S phase progressively reduced and eventually fell to zero, whereas cells in the G_2M phase increased (Fig. 4). DDP was approximately 20-fold more active than DWA2114R in producing cell cycle perturbation. These findings are in accord with the platinum sensitivity of these cell lines (Fig. 1).

Differences in cell cycle perturbation caused by DDP among cell types In PC-6 cell line, with increasing DDP concentration the percentage of early-middle S phase cells gradually declined and eventually fell to zero. In PC-3 and PC-10 cell lines, however, with increasing DDP concentrations the early-middle S shifted gradually to the late S and then returned to the early S phase without G_2M accumulation (data not shown). Computerized analyses of these bivariate cytograms (Fig. 5) of BrdUrd and DNA showed that, in the case of PC-6 cell line, increase in DDP concentration resulted in a gradual increase in G_2M population ratio and decrease in S, though the G_1 cell ratio did not decrease. At higher concentrations, however, increases in G_2M phase cells and decreases in S phase cells were observed with concomitant decreases in G_1 cells. In the case of PC-3, the G_1 population ratio decreased gradually with increasing DDP concentration, G_2M cells remained stable and at higher DDP concentrations declined, and only S cells increased. A similar pattern was seen with PC-10 cell line (data not shown).

Differences in cell cycle perturbation caused by DWA-2114R among cell types Although the activity of DWA-2114R was less than that of DDP, cell cycle perturbations produced by the two drugs were essentially similar. Thus, in PC-6, cells in early-middle S disappeared at concentrations 20-fold higher than in the case of DDP. Transition of PC-3 cells from the early-middle S to the late S also reguired higher DWA2114R concentrations as compared with DDP. The compound produced no major changes in the PC-10 histogram (data not shown). Computerized analyses of the bivariate cytograms of PC-6 cells revealed changes similar to those seen with DDP: an accumulation of G_2M cells and diminution of cells in the S phase in a concentration-dependent manner and a decrease in G_1 cells at high DWA2114R concentrations (Fig. 6). In PC-3 cell line, however, hardly any major change occurred within the concentration range studied.

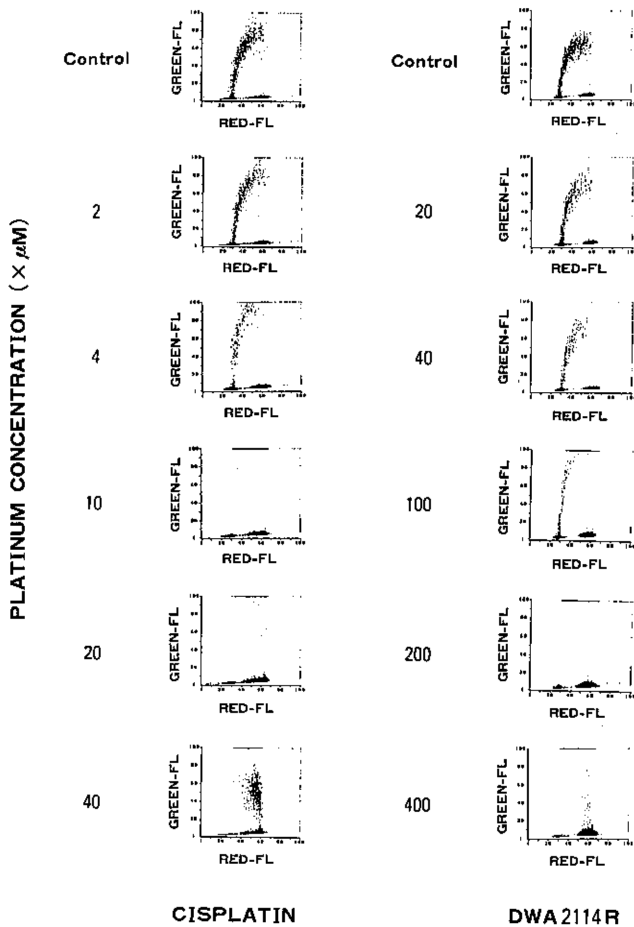


Fig. 4. Comparison of PC-6 cell cycle perturbation caused by cisplatin and by DWA2114R. PC-6 cells were exposed to cisplatin or DWA2114R for 1 h. BrdUrd was added 20 min before the end of 24 h reculture.

Computerized analyses of PC-10 cells gave similar results to those seen with PC-3 cells (data not shown).

Relationship between exposure time and drug concentration for cell cycle perturbation In order to produce the same degree of cell cycle perturbations, a longer exposure time was required if a lower concentration of DWA-2114R was used. Essentially, (exposure time) × (drug concentration) was constant (Fig. 7). A similar relationship was observed with DDP (data not shown). These results suggest that both DWA2114R and DDP act in a cell cycle phase-nonspecific manner.

Comparison of Ki-67 Ag appearance patterns after exposure to DDP vs. DWA2114R Ki-67 Ag appearance patterns of PC-10 cells 24 h after drug exposure for 1 h are shown in Fig. 8. When treated with increasing concentrations of DWA2114R, G_1 cells bearing high Ki-67

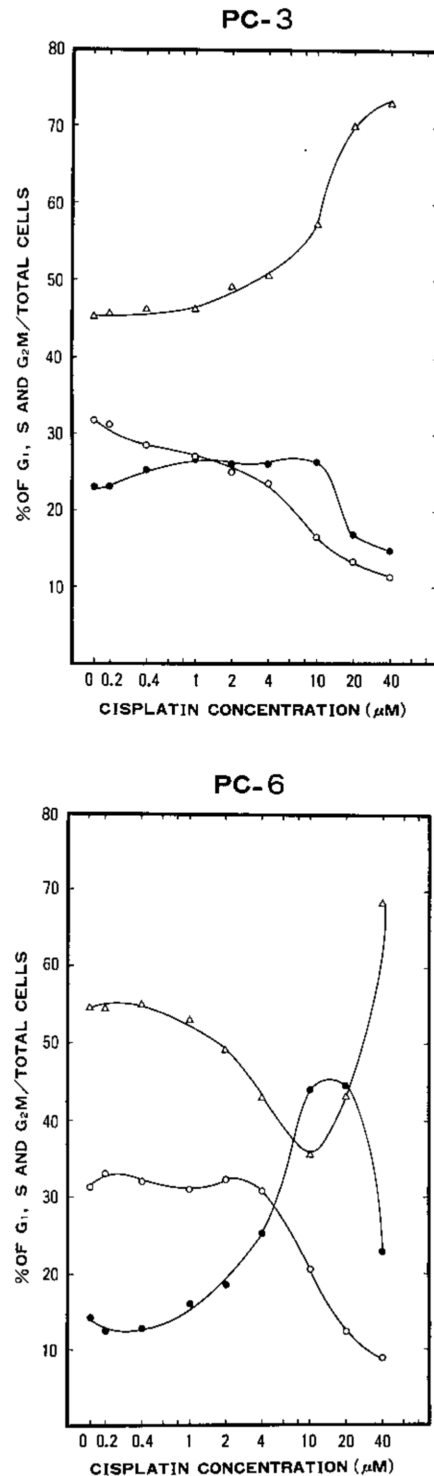


Fig. 5. Differences in PC-3 and PC-6 cells of cell cycle perturbation produced by cisplatin. Computerized analyses were performed using region statistics columns according to the manufacturer's instructions. ○, △ and ● indicate percent of G_1, S and G_2M/total number of cells, respectively.

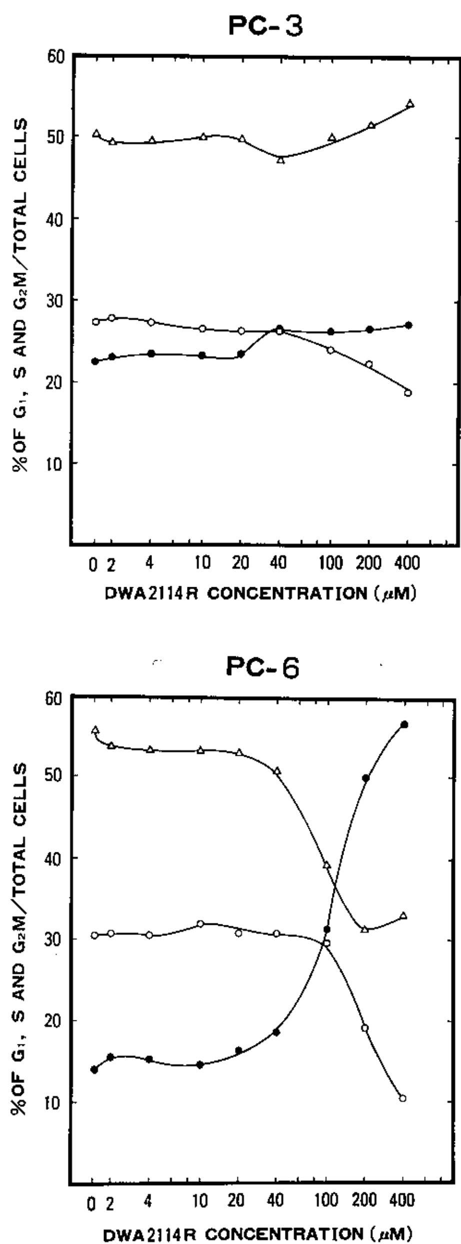


Fig. 6. Differences in cell cycle perturbation of PC-3 and PC-6 cells produced by DWA2114R. Computerized analyses were performed using region statistics columns. ○, △ and ● indicate percent of G_1, S and G_2M/total number of cells, respectively.

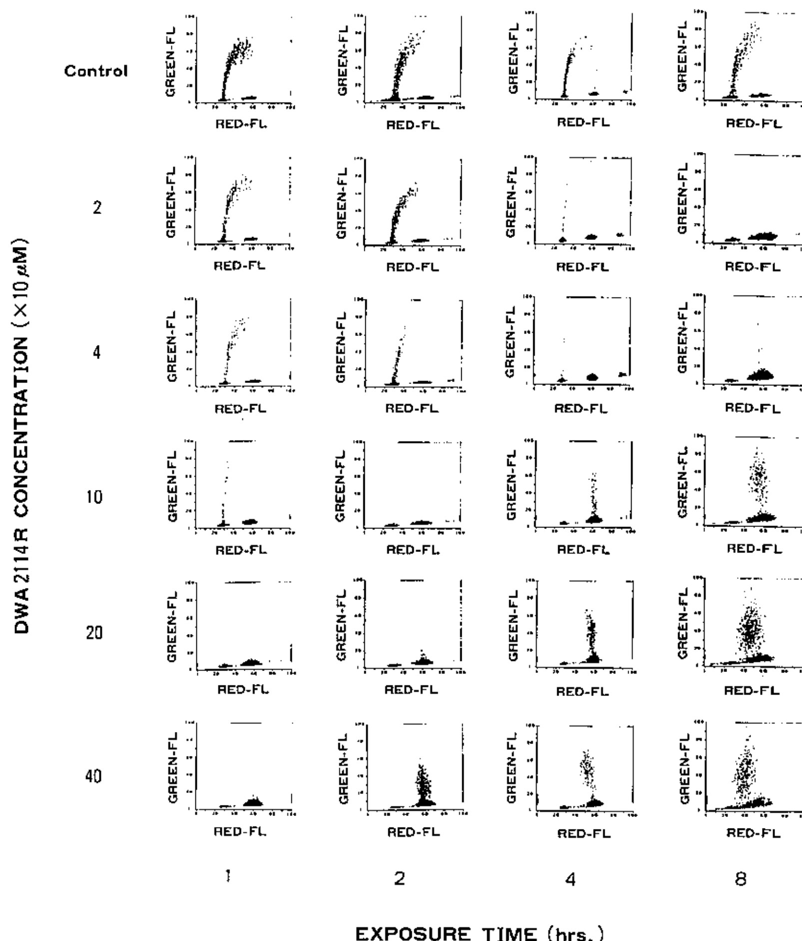


Fig. 7. Relationship between exposure time and drug concentration in cell cycle perturbation of PC-6 small cell carcinoma cells caused by DWA2114R. Each bivariate cytogram was obtained 24 h after exposure to graded concentrations of DWA2114R for various times. Cytograms showing similar patterns of cell cycle perturbation were chosen and the relationships between exposure time and drug concentration in the cytograms were evaluated.

Ag decreased initially and then those bearing low Ki-67 Ag decreased. As compared with DWA2114R, DDP produced decreases in either G_1 cells bearing high Ki-67 Ag or those bearing low Ki-67 Ag (Fig. 8). This observation can be better illustrated in computerized analyses, which showed treatment with DDP resulted in a linear decrease in the low Ki-67 population and a relative equilibrium of high Ki-67 Ag population (Fig. 9). In contrast, in DWA2114R-treated cells the relationship between low and high Ki-67 populations was biphasic and the curves were symmetrical. With increasing DWA2114R concentrations the numbers of both cell populations gradually decreased. Because PC-6 had fewer G_1 cells bearing low Ki-67 Ag and PC-3 cells were less sensitive to both drugs, changes in the manifestation of Ki-67 Ag in PC-3 and PC-6 cells were not so characteristic as those in PC-10 cells (data not shown).

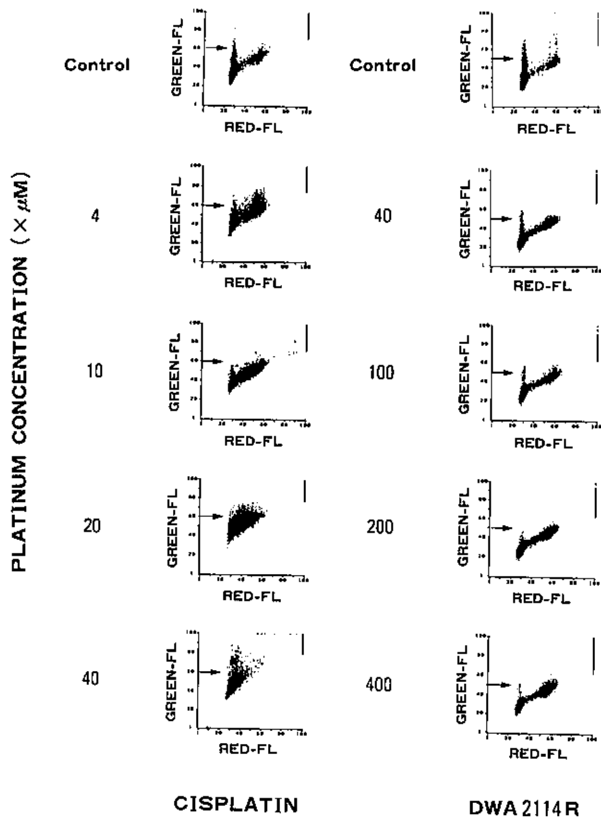
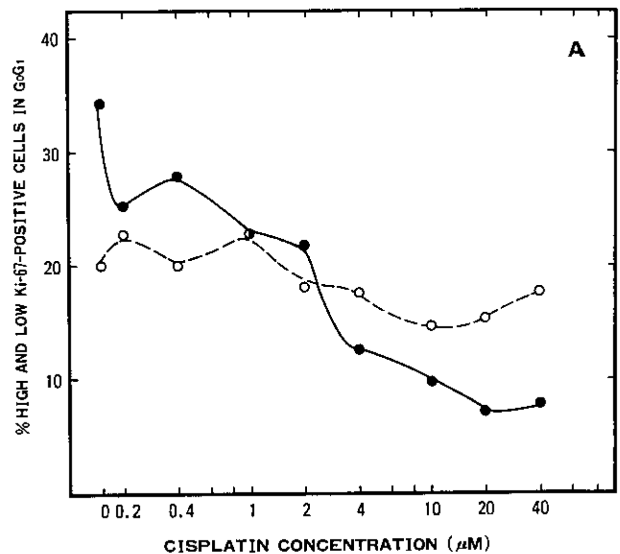


Fig. 8. Comparison of Ki-67 Ag appearance pattern of PC-10 squamous cell carcinoma cells exposed to cisplatin and to DWA2114R. An arrow in each bivariate cytogram of Ki-67 Ag (GREEN)/DNA (RED) points to the portion of G_1 cells with high Ki-67 Ag level.



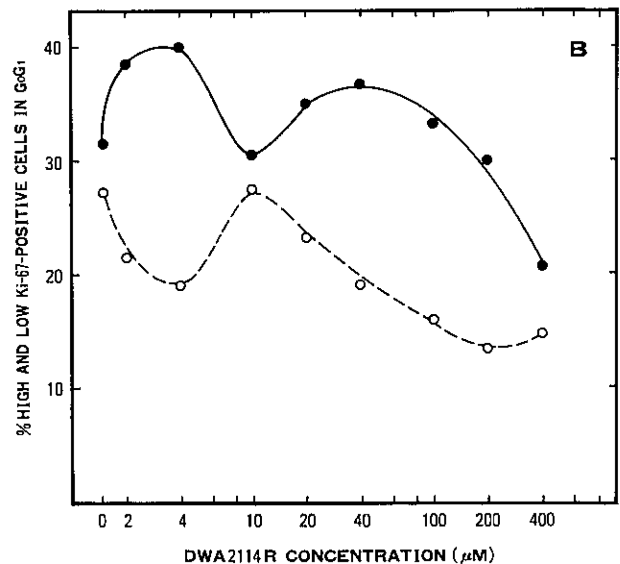


Fig. 9. Effects of cisplatin and DWA2114R on Ki-67 Ag appearance pattern of PC-10 squamous cell carcinoma cells in the G_1. Computerized analyses of Fig. 8 were performed using region statistics columns. Cells bearing high Ki-67 Ag are indicated by ○ and low Ki-67 Ag by ●.

Effects of DDP and DWA2114R on incorporation of Rho by live mitochondria Treatment of PC-6 and PC-10 cells with DDP caused depression of Rho uptake (Fig. 10), whereas treatment with DWA2114R caused no change even at 1 mM (data not shown). Mitochondria of PC-3 adenocarcinoma cells appeared to be widely distributed and were not affected by either DDP or DWA2114R.

DISCUSSION

Our observations may be summarized as follows. a) Simultaneous exposure of cells to DDP and BrdUrd resulted in decreases in fluorescence intensity, i.e., in the amount of BrdUrd incorporated into single-stranded DNA. b) DDP appears to be approximately 20-fold more active than DWA2114R in producing cell cycle perturbation. c) In PC-6 cell line, DDP produced decreases in S phase cells and accumulation of cells in the G_2M phase, whereas in PC-10 and PC-3 cell lines, DDP produced S phase cell accumulation. Weaker but similar changes occurred with DWA2114R. d) The high Ki-67 Ag cell population decreased when treated with either DDP or DWA2114R, but DDP reduced low Ki-67 Ag population more than DWA2114R. e) In PC-10 and PC-6 cells, DDP suppressed Rho incorporation into live mito-

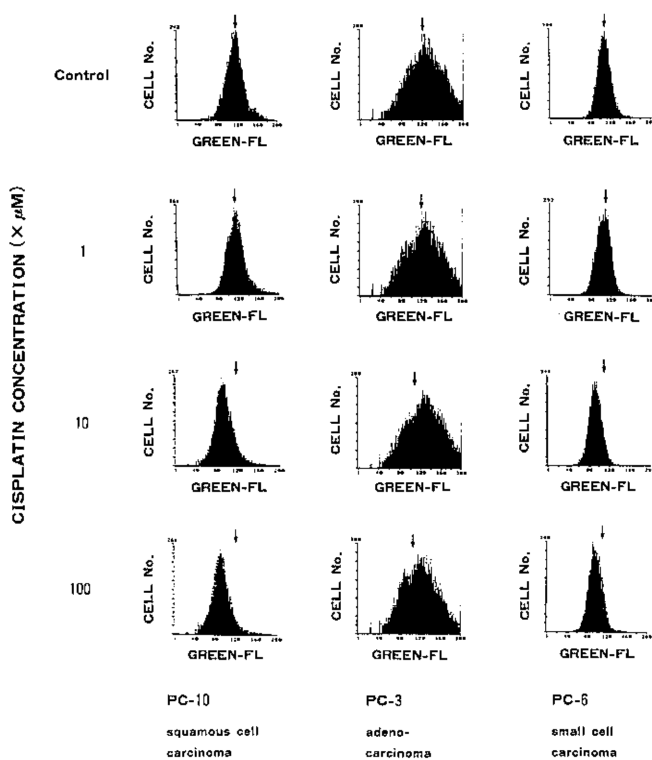


Fig. 10. Single parameter log fluorescence histograms for rhodamine 123 incorporated into live mitochondria of lung carcinoma lines exposed to cisplatin. The vertical axis indicates cell number and the horizontal axis shows green fluorescence intensity of rhodamine 123 in each histogram. Arrows indicate mean channel numbers of green fluorescence intensity of control cells. Peak channel numbers of the gree fluorescence of PC-10 cells are 114, 114, 91 and 91 from the top to bottom histograms respectively and those of PC-6 cells are 105, 104, 92 and 91 from the top to bottom histograms, respectively.

chondria, whereas DWA2114R produced no change in Rho incorporation. PC-3 cells were not affected by either DDP or DWA2114R.

Flow cytometry was reported to be a useful tool in analyzing cell cycle perturbation caused by anticancer drugs.[13-15] We observed that DDP or DWA2114R produced suppression of BrdUrd incorporation. Possible mechanisms of this suppression may include competition between BrdUrd and platinum at the membrane and/or intracellular transport site as well as at DNA cross-linking sites. We observed that brief (20 min) drug exposure resulted in disturbance of cell cycle transition only at high concentrations of DDP, at which the suppression of BrdUrd incorporation occurred. Differences in the affinity of BrdUrd and platinum compounds for DNA may be an important factor to explain these observations. The affinity of platinum for DNA has been measured in a cell-free system[16,17]; however, the affinity of platinum for DNA in the whole cell has not been reported. We found that the two platinum compounds could be distinguished by measurement of suppression of BrdUrd incorporation. Measurement of competition of platinum with known agents may afford more information about platinum binding in living cells.

Sorenson and Eastman studied the DNA histogram and ^{3}H-thymidine uptake and reported that DDP produced arrest in the G_2 phase in Chinese hamster ovary cells sensitive to DDP, whereas DDP produced inhibition of DNA synthesis through the S phase in repair-proficient cells.[18] In analysing the S phase cells by means of simultaneous staining with BrdUrd and PI, we found that DDP produced decreases in S phase population as well as cell accumulation in G_2 in PC-6 cells sensitive to both DDP and DWA2114R. This observation indicates that in PC-6 cells shortening of the S phase and simultaneous prolongation of the G_2 phase occurred. In the case of PC-3 and PC-10 cells, which are less sensitive to DDP and DWA2114R than PC-6 cells, DDP and DWA2114R produced shifts from late S to early S with increasing drug concentration. Thus, platinum appears to produce either S phase prolongation or shortening depending on the cell type. We suppose that the S phase shortening is due to dominant interstrand cross-links and the S phase prolongation results from dominant intrastrand cross-linking. The degree of interstrand cross-linking, as well as bis-N^7-guanylplatinum intrastrand cross-links,[19,20] has been reported to correlate quantitatively with cytotoxicity.[21,22] Since our results suggest that S phase shortening plays a more important role in platinum-induced cytotoxicity, further experiments on the relationship between cross-linking and cell cycle perturbation are required.

We found that C (drug concentration) $\times t$ (exposure time) is essentially constant, based on observations of degree of cell cycle perturbation. It was reported that the cell killing effects of drugs lacking cell cycle phase specificity were dependent on $C\times t$, and DDP belongs to this class of compounds.[23] From these observations, both DWA2114R and DDP can be categorized as cell cycle phase-nonspecific agents.

Ki-67 Ag levels are known to be negative in the G_0 phase and to increase with cell cycle progression.[9,10,24] Changes in Ki-67 level in G_1 cells have not been well established, however. We found that there were two types of G_1 population: G_1 cells having high levels of Ki-67 Ag were more sensitive to DWA2114R than those with low Ki-67 Ag levels. Two mechanisms can be considered to explain this phenomenon. a) Because of prolongation of the S or the G_2 phase the numbers of cells entering G_1 decreased. b) There were two pathways in

G_1; in one, high Ki-67 G_1 cells rapidly enter the S phase and in the other, low Ki-67 G_1 cells remain longer in G_1. Our observations indicate that cells remaining in G_1 and with low Ki-67 Ag levels are less sensitive to DWA2114R. Ki-67 Ag may serve as an important marker for platinum sensitivity. It is of note that normal cells in general bear little or no Ki-67 Ag.[8] DDP's activity against low Ki-67 cells suggests enhanced effects on normal tissues or enhanced toxicity as compared to DWA2114R. Further studies are in progress to substantiate these observations using other cell lines.

Mitochondria may be another target of DDP in certain cells.[25] In our study, DDP affected the mitochondria of both PC-6 and PC-10 cells; however, DWA2114R did not affect them even at 20-fold higher concentration than that of DDP. Whether the effect of DDP on mitochondria has any relevance to its biological activity is unknown. PC-3 cells showed a wide distribution of Rho-positive cells and were not affected by DDP. Generally, lung adenocarcinoma has been regarded as DDP-insensitive.[26] It would be of interest to study whether the wide distribution of Rho-positive cells signals insensitivity to platinum in the adenocarcinoma. In addition, differences in the ability of DDP and DWA2114R to inhibit incorporation of Rho into mitochondria may be useful in distinguishing certain platinum compounds.

The purpose of this study was to characterize the differences in the action mechanisms of DDP and DWA-2114R by flow-cytometric means. As described above, DDP rapidly interacts with DNA. This interaction either causes a shortened S and a prolonged G_2 phase or results in a shift of the S phase from the late S to the early S. Besides, the number of G_1 cells containing high Ki-67 Ag levels was reduced and the number with lower Ki-67 Ag levels remained unchanged. DDP also reacted with mitochondria. DWA2114R may have a lower affinity for DNA as compared with DDP, since neither suppression of BrdUrd incorporation during brief incubation nor any effect on mitochondria was observed. It is likely that these differences are ralated to DWA2114R's biological activity.

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REFERENCES

1) Guarino, A. M., Miller, D. S., Arnold, S. T., Pritchard, J. B., Davis, R. D., Urbanek, M. A., Miller, T. J. and Litterst, C. L. Platinate toxicity: past, present, and prospects. *Cancer Treat. Rep.*, **63**, 1475–1483 (1979).
2) Von Hoff, D. D., Schilsky, R., Reichert, C. M., Reddick, R. L., Rozencweig, M., Young, R. C. and Muggia, F. M. Toxic effects of *cis*-dichlorodiammine-platinum(II) in man. *Cancer Treat. Rep.*, **63**, 1527–1531 (1979).
3) Ota, K. New platinum derivatives in Japan. *In* "Cancer Chemotherapy: Challenges for the Future," Vol. 2, ed. K. Kimura, K. Ota, R. B. Harberman and H. Takita, pp. 329–338 (1987). Excerpta Medica, Tokyo.
4) Koizumi, M., Honda, M., Morikawa, K., Endoh, K., Matsumoto, T., Akamatsu, K. and Matsui, H. Antitumor activity of a new platinum cytostatic, DWA2114R. *In* "Proceedings of the Fifth International Symposium on Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy," ed. M. Nicolini, pp. 695–699 (1987). Martinus Nijhoff Publishing, Boston.
5) Goz, B. The effects of incorporation of 5-halogenated deoxyuridines into the DNA of eukaryotic cells. *Pharmacol. Rev.*, **29**, 249–272 (1978).
6) Szybalski, W. X-ray sensitization by halopyrimidines. *Cancer Chemother. Rep.*, **58**, 539–557 (1974).
7) Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J. and Melamed, M. R. Interactions of rhodamine 123 with living cells studied by flow cytometry. *Cancer Res.*, **42**, 799–806 (1982).
8) Darzynkiewicz, Z., Staiano-Coico, L. and Melamed, M. R. Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. *Proc. Natl. Acad. Sci. USA*, **78**, 2383–2387 (1981).
9) Gerdes, J., Lemke, H., Baisch, H., Waker, H-H., Schwab, U. and Stein, H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immnol.*, **133**, 1710–1715 (1984).
10) Schwarting, R., Gerdes, J., Niehus, J., Jaeschke, L. and Stein, H. Determination of the growth fraction in cell suspensions by flow cytometry using the monoclonal antibody Ki-67. *J. Immunol. Methods*, **90**, 65–70 (1986).
11) Inoue, S., Ito, M., Mizuno, S., Isobe, H., Miyamoto, H., Kawakami, Y. and Ohnuma, T. Simultaneous flow cytometric detection of nuclear DNA and tumor-associated antigens in lung cancers. *Anal. Quant. Cytol. Histol.*, **10**, 243–250 (1988).
12) Schutte, B., Reynders, M. M. J., van Assche, C. L. M. V. J., Hupperets, P. S. G. J., Bosman, F. T. and Blijham, G. H. An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry. *Cytometry*, **8**, 372–376 (1987).
13) Frankfurt, O. S. Flow cytometric analysis of DNA damage and the evaluation of cytotoxicity of alkylating agents. *Cancer Res.*, **47**, 5537–5541 (1987).
14) Tsurusawa, M., Niwa, M., Katano, N. and Fujimoto, T. Flow cytometric analysis by bromodeoxyuridine/DNA assay of cell cycle perturbation of methotrexate-treated mouse L1210 leukemia cells. *Cancer Res.*, **48**, 4288–4293 (1988).
15) Riccardi, A., Danova, M., Wilson, G., Ucci, G., Dörmer,

P., Mazzini, G., Brugnatelli, S., Girino, M., McNally, N. J. and Ascari, E. Cell kinetics in human malignancies studied with *in vivo* administration of bromodeoxyuridine and flow cytometry. *Cancer Res.,* **48,** 6238–6245 (1988).
16) Fichtinger-Schepman, A. M. J., van Oosterom, A. T., Lohman, P. H. M. and Berends, F. *cis*-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of *cis*-diamminedichloroplatinum(II). *Cancer Res.,* **47,** 3000–3004 (1987).
17) Dijt, F. J., Fichtinger-Schepman, A. M. J., Berends, F. and Reedijk, J. Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair deficient human fibroblasts. *Cancer Res.,* **48,** 6058–6062 (1988).
18) Sorenson, C. M. and Eastman, A. Influence of *cis*-diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. *Cancer Res.,* **48,** 6703–6707 (1988).
19) Eastman, A. Reevaluation of interaction of *cis*-dichloro-(ethylenediamine) platinum (II) with DNA. *Biochemistry,* **25,** 3912–3915 (1986).
20) Scherman, S. E., Gibson, D., Wang, A. H-J. and Lippard, S. J. X-ray structure of the major adduct of the anticancer drug cisplatin with DNA: *cis*-[Pt$(NH_3)_2$ {d(pGpG)}]. *Science,* **230,** 412–417 (1985).
21) Bedford, P., Walker, M. C., Sharma, H. L., Perera, A., McAuliffe, C. A., Masters, J. R. W. and Hill, B. T. Factors influencing the sensitivity of two human bladder carcinoma cell lines to *cis*-diamminedichloroplatinum(II). *Chem.-Biol. Interact.,* **61,** 1–15 (1987).
22) Teicher, B. A., Holden, S. A., Kelley, M. J., Shea, T. C., Cucchi, C. A., Rosowsky, A., Henner, W. D. and Frei, E., III. Characterization of a human squamous carcinoma cell line resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res.,* **47,** 388–393 (1987).
23) Ozawa, S., Sugiyama, Y., Mitsuhashi, J. and Inaba, M. Kinetic analysis of cell killing effect induced by cytosine arabinoside and cisplatin in relation to cell cycle phase specificity in human colon cancer and Chinese hamster cells. *Cancer Res.,* **49,** 3823–3828 (1989).
24) Sasaki, K., Murakami, T., Kawasaki, M. and Takahashi, M. The cell cycle associated changes of the Ki-67 reactive nuclear antigen expression. *J. Cell Physiol.,* **133,** 579–584 (1987).
25) Singh, G. and Maniccia-Bozzo, E. Evidence for lack of mitochondrial DNA repair following *cis*-dichlorodiammineplatinum treatment. *Cancer Chemother. Pharmacol.,* **26,** 97–100 (1990).
26) DeVita, V. T. Principles of chemotherapy. *In* "Cancer: Principles and Practice of Oncology," 2nd Ed., ed. V. T. DeVita, S. Hellman and S. A. Rosenberg, pp. 257–285 (1985). L. B. Lippincott Co., Philadelphia.