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Effect of Cisplatin on Cell Death and DNA Crosslinking in Rat Mammary Adenocarcinoma *in vitro*

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The pharmacodynamic effects of *cis*-diamminedichloroplatinum(II) (CDDP) *in vitro* have been reported, but the dosage and exposure time *in vitro* have not been based on clinical observations of the drug's actions *in vivo*. In this study, the authors attempted to evaluate the pharmacodynamic effects of CDDP *in vitro* in terms of cell survival and DNA crosslinking by simulating unbound CDDP administration at varying concentrations to a rat mammary adenocarcinoma line (known as line 66). CDDP exposure was conducted by both constant concentration procedures and a simulated *in vivo* procedure. Colony formation assay for the surviving fraction and alkaline elution assay for DNA crosslink measurement were performed in order to evaluate the pharmacodynamics of CDDP. Cell survival was a function of the area under the drug concentration time curve (AUC) of unbound CDDP ($R^2 = 0.77$, $P < 0.002$) for all drug exposure procedures as analyzed by the analysis of covariance test. There was a strong correlation between the surviving fraction and the crosslink index of the total amount of DNA crosslinks ($R^2 = 0.85$, $P < 0.0005$). Both the total amount of DNA-DNA crosslinks and the DNA-protein crosslinks, of which the latter were dominant, were affected not by the exposure procedures, but by the AUC value ($P < 0.002$). The thresholds of cytocidal effect were 1.59 mg·h/l for the AUC and 0.008 for the crosslink index. The pharmacodynamic effects *in vitro* by simulated *in vivo* exposure were identical to those of constant.

Key words: pharmacodynamics, pharmacokinetics, simulation, cisplatin, crosslink

Many kinds of anti-cancer agents are in wide clinical use and there have been many papers published on the *in vitro* cytotoxity of these anti-cancer agents. However, the dosages of drugs in some of these studies are different from those used in actual clinical practice (usually the *in vitro* dosages are artificially high). In general, the higher the drug concentration, the clearer the experimental outcome will be. However, in clinical practice, such high dosages cannot be given to patients because of severe toxicity. Thus, we felt that the experimental parameters of *in vitro* studies, such as drug concentration and exposure time, should approximate the *in vivo* pharmacokinetic parameters as closely as possible. In other words, it is of value to simulate *in vivo* conditions in experiments *in vitro* so that the results of *in vitro* experiments can be applied to patients as efficiently as possible in order to improve existing clinical drug regimens.

In gynecologic oncology, *cis*-diamminedichloroplatinum(II) (CDDP) is one of the most important drugs for chemotherapy. The pharmacodynamics of platinum analogues are said to be dependent on the area under the drug concentration time curve (AUC) value. This is based on both experimental data *in vitro* and a mathematical model (1). On the other hand, clinical observations suggest that the anti-tumor effect of CDDP is not influenced by the dosage (2), which theoretically is proportional to the AUC value if the drug shows pharmacological linearity. This suggests that there is some discrepancy between the experimental data supported by the model and actual clinical data. CDDP binds irreversibly to plasma and cellular components and protein-bound CDDP is not able to form CDDP-DNA adducts (3). Moreover, cell kill is determined not by the rate of input of CDDP, but by the magnitude of exposure to unbound

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CDDP (3). Therefore, it is of importance to investigate that the *in vitro* pharmacodynamic effects of unbound CDDP (in simulated *in vivo* exposure conditions) are identical to those of constant concentration.

To simulate the pharmacodynamics of CDDP *in vitro*, it is important that the drug concentration *in vitro* be simulated with the active form of the drug in a time-dependent manner, namely, $Ae^{-\alpha t} + Be^{-\beta t}$ as in the case of the two-compartment model. Ma *et al.* demonstrated in a simulated *in vivo* experiment (by adding albumin to the medium to eliminate unbound CDDP *in vitro*) that there was a log-linear relationship between the AUC of unbound CDDP and cell survival (3). Their study, however, did not evaluate protein-bound CDDP within cells. In the present study, the authors investigated cell survival and DNA crosslinks of unbound CDDP *in vitro* using a rat adenocarcinoma cell line by *in vivo* pharmacokinetic simulation, in which the CDDP concentration varied relative to the constant concentration exposure.

Materials and Methods

Cell growth and maintenance. Cells derived from a rat mammary adenocarcinoma (designated cell line 66) were grown in modified McCoy's 5A medium (GIBCO, Grand Island, NY, USA) supplemented with 0.05% L-glutamine (GIBCO), 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate (GIBCO) (4) and 10% v/v heat-inactivated fetal calf serum (GIBCO) in humidified air enriched with 5% CO_2 at 37°C. Exponentially proliferating cells grown for 3-4 days were used.

Drug exposure. CDDP was kindly provided by Bristol-Myers-Squibb K.K. (Tokyo, Japan). Since 97% of CDDP binds to protein in sera after 24 h (3) and the active form is unbound CDDP, CDDP was dissolved at 37°C in modified McCoy's 5A medium without sera prior to CDDP exposure so that all of the dissolved CDDP would be unbound in the medium. The solution was warmed at 37°C so that all of the exposures could be performed at 37°C. Four T75 flasks (Becton-Dickinson, Franklin Lakes, NJ, USA) containing approximately 6×10^6 cells in 15 ml of medium per flask were used in each experiment.

Simulated exposure. Simulated exposure was based on clinical pharmacokinetic parameters. The AUC value with the ordinal CDDP dose (120 mg/m^2) was 4.8 mg·h/l in continuous 5-day infusion and 9.6 mg·h/l in 30-min infusion of the AUC value of unbound CDDP, respectively (5). Vermorken *et al.* reported that the level of unbound CDDP was relatively low 12 h after the end of less than 3-h infusion (2). The simulated *in vivo* exposure mimics 1-h administration of CDDP. All CDDP exposure times were set at 12 h. The AUC value of unbound CDDP was set at 2, 4 and 8 mg·h/l. The time-course of change in the unbound CDDP concentration after the first hour was simulated *in vitro* so that the $T\alpha_{1/2}$ (0.60 h^{-1}) and $T\beta_{1/2}$ (2.50 h^{-1}), settings were the same as those *in vivo* (Fig. 1). The concentration of the first hour unbound CDDP exposure was set to mimic the predicted concentration *in vivo* to achieve the target AUC value. Other parameters such as maximum concentration were calculated, based on the AUC value and half-lives, using an original program by Mathematica (Wolfram Research, Inc., Champaign, IL, USA) on a Macintosh computer (Apple Computer, Inc., Cupertino, CA, USA). In the case of 8 mg·h/l of the AUC value of unbound CDDP, the CDDP concentrations were 1.389, 4.000, 1.259, 0.397, 0.227, 0.131, 0.075, 0.043 mg/l during 0-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-10, 10-12 h, respectively. The maximum unbound CDDP concentration, 4 mg/l, was identical to that of short-term infusion *in vivo* (5). To obtain other concentration/time lists for 2 and 4 mg·h/l of the AUC values, the list of 8 mg·h/l was divided by 4 and 2, respectively.

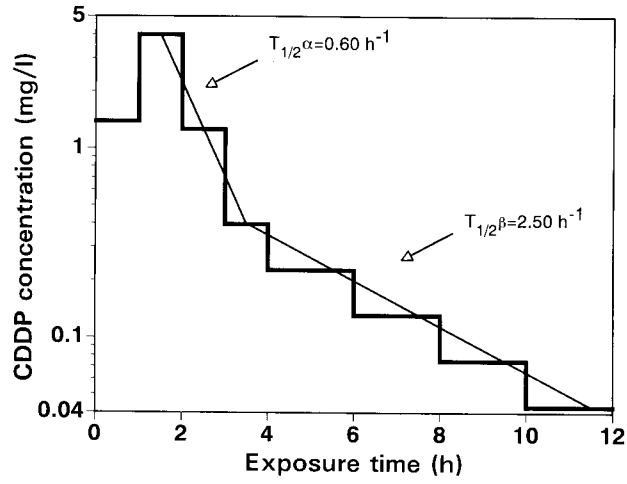

Fig. 1 Simulated *in vivo cis*-diamminedichloroplatinum(II) (CDDP) exposure. The *in vivo* CDDP concentration is simulated *in vitro*, based on a clinical study (1). Data for the 8 mg·h/l of the area under the drug concentration time curve (AUC) value is shown. In experiments with other AUC values, the exposure time and half-life times of both the α and β phases were kept constant.

Constant concentration exposure The maximum unbound CDDP concentration obtained by short-time infusion *in vivo* was 4 mg/l (5), which was defined as high-dose exposure in this study. Since the AUC values investigated should be 2, 4 and 8 mg·h/l, an exposure time at 4 mg/l as the high dose was set at 0.5, 1.0 and 2.0 h, respectively. Since unbound CDDP is eliminated and is too low to detect after 12 h *in vivo*, the low-dose concentration was set at 0.67 mg/l, which achieved 8 mg·h/l of the AUC in 12 h. Therefore, the exposure time at 0.67 mg/l was set at 3, 6 and 12 h, resulting in 2, 4 and 8 mg·h/l of the AUC value, respectively.

With both methods of CDDP exposure, after exposure, the cells were rinsed three times with CDDP-free modified McCoy's 5A medium with 10 % v/v fetal calf serum at 37°C. The cells were then trypsinized and resuspended in the medium at 37°C.

Colony formation. Colony formation assay was used as a survival assay in this study. Briefly, 100–5000 of the cells suspended in modified McCoy's medium with 10 % v/v fetal calf serum were seeded into culture dishes 5 cm in diameter. Surviving colonies were counted after 12–14 days.

DNA analysis. DNA crosslinking was measured by the alkaline elution assay described by Miyagi *et al.* (4) with modifications. Briefly, the treated cells were incubated at 37°C for 4 h after the rinse followed by the trypsinization. Cell suspensions at 37°C were irradiated with 7.5 Gy of X-rays with an irradiator, MBR-1520 (Hitachi-Medico, Tokyo, Japan), then cooled immediately in ice. After 3×10^6 of the cells for each channel were loaded onto polycarbonate filters (Costar Scientific Corp., Cambridge, MA, USA) in Swinnex filter holders, the cells were lysed with or without proteinase K (Merk, Darmstadt, Germany) in the lysis solution. Next, 33 ml of a solution of tetrapropylammonium hydroxide, pH 12.3, was overlayered and pumped through the filter at a rate of 0.0389 ml/min. At least 3 channels were used for each cell suspension specimen and all of the DNA analyses were repeated 3 times.

Analysis of alkaline elution data. The amounts of DNA from cells on the filters were calculated as the percentage of the amount retained after the elution. The frequency of CDDP-induced DNA crosslinking in this study was calculated using the following formula (6):

$$CLI_{total} = [(1 - R_0)/(1 - R_{CDDP})]^{1/2} - 1,$$

$$CLI_{DPC} = [(1 - R_0)/(1 - R_{CDDPPK})]^{1/2} - 1,$$

$$CLI_{ISC} = CLI_{total} - CLI_{DPC},$$

in which CLI_{total} is the crosslink index of DNA crosslinking of CDDP-treated cells; CLI_{DPC} is the crosslink index of DNA-protein crosslinking; CLI_{ISC} is the crosslink index of DNA-interstrand crosslinking; R_0 is the relative retention of irradiated cells without CDDP exposure; R_{CDDP} is that of CDDP-treated cells without proteinase K after 7.5 Gy irradiation; R_{CDDPPK} is that of CDDP-treated cells with proteinase K after 7.5 Gy irradiation.

Statistical analysis. The values were analyzed using the analysis of variance test with a subsequent post hoc test and the analysis of covariance test.

Results

Cell survival and DNA crosslink formation were not affected by serum-free incubation for 12 h (data not shown). Cell survival was a function of time in the constant concentration exposure experiments and was a function of the AUC value irrespective of the various drug exposure methods (Fig. 2). Colony-forming efficiency of untreated cells was 72.6 ± 3.9 % (mean ± S.E.). Strong correlation was observed between the logarithm of the colony formation efficiency values and the AUC values ($R^2 = 0.77$, $P < 0.002$) and the estimated threshold of the AUC value for cell kill was 1.59 mg·h/l.

The maximum value of DNA crosslink formation was observed around 4–6 h after the removal of CDDP (Fig. 3). Therefore, the alkaline elution assay was performed 4 h after the removal of CDDP in this study. There was a strong correlation between colony formation efficiency and crosslink index ($R^2 = 0.85$, $P < 0.0005$) shown in Fig. 4. The calculated threshold of the crosslink index for cell killing was 0.008. These findings strongly suggested that the cell killing effect is dependent on the amount of DNA crosslinks and is not affected by drug exposure methods.

Fig. 5 shows the kinetics of DNA crosslink formation as a function of the AUC value. DNA-protein crosslinks involved 68.3 ± 13.1 % (mean ± S.D.) of the total amount of DNA crosslinks (data not shown). The greater the AUC, the higher the total crosslink index became, though a significant increase was not observed at lower AUC values in the 2–4 mg·h/l range, which is less than the amounts usually used in clinical practice. It is likely that crosslink formation under 4 mg·h/l of the AUC value is under the detection threshold of the alkaline elution assay. The crosslink indices of both the total number of DNA crosslinks and DNA-protein crosslinks at 2 mg·h/l were not statistically different from those of 4 mg·h/l,

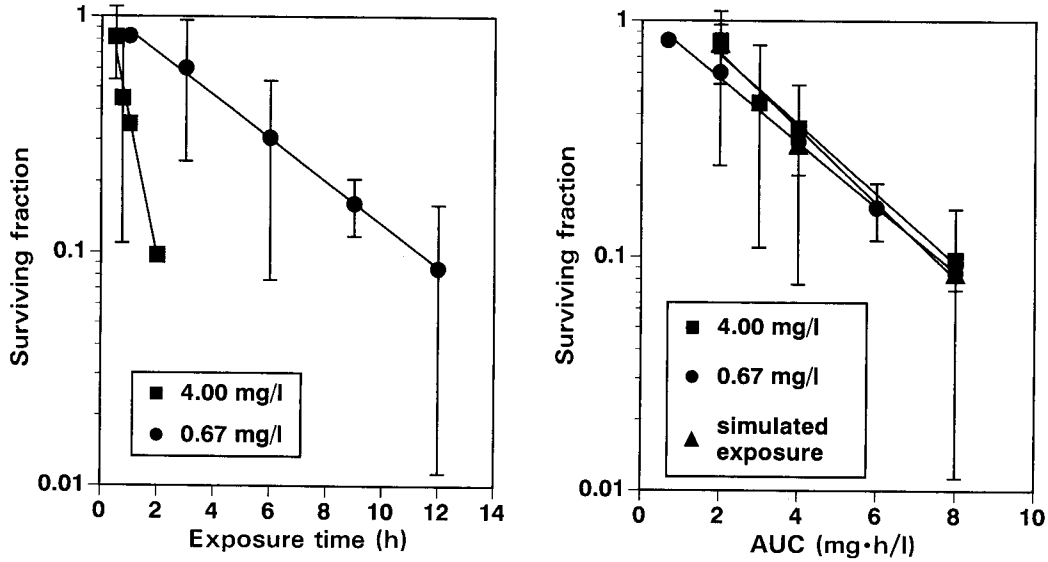

Fig. 2 Colony formation efficiency as a function of time in 2 types of constant drug concentration exposure methods (left panel) and as a function of the area under the drug concentration time curve (AUC) value in 3 methods of drug exposure (right panel). No statistical difference in the colony formation efficiency was observed in the right panel.

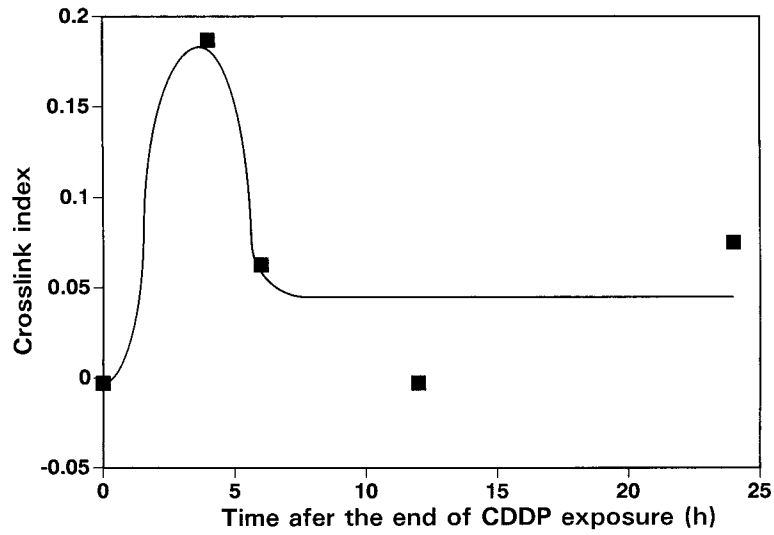

Fig. 3 DNA repair kinetics at 4 mg/l of *cis*-diamminedichloroplatinum(II) (CDDP) constant exposure evaluated by the alkaline elution assay. The maximum DNA crosslink formation was observed at 4 h after the end of drug exposure.

irrespective of the drug exposure method used. The one-way analysis of variance test revealed that the drug exposure procedures did not affect the amount of DNA crosslink formation. The test also revealed that both the total amount of DNA crosslink formation and DNA-protein crosslink formation were not affected at all by the drug exposure methods but were affected by the AUC value ($P < 0.002$ each for both crosslink formation rates). The subsequent post hoc test revealed that there were statistically significant differences between the crosslink index value at 8 mg·h/l and those of both 2 and 4 mg·h/l ($P < 0.005$). The AUC value appears to affect inter-strand crosslink formation almost as significantly as DNA-protein crosslink formation ($P = 0.0639$).

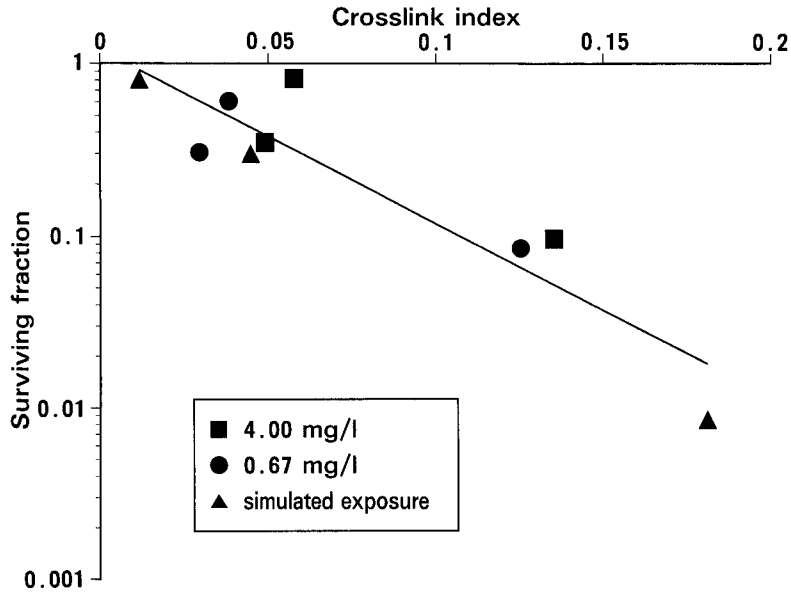

Fig. 4 Correlation between DNA crosslinking and cell survival. The logarithm of colony formation efficiency is a function of the DNA crosslink index (CLI), irrespective of exposure method. ($\ln(Y') = 0.185(\pm 0.343) - 23.16(\pm 3.71)x$, $R^2 = 0.848$, $P = 0.0004$). The amount of DNA crosslinking assayed by the alkaline elution technique allows one to predict cell survival.

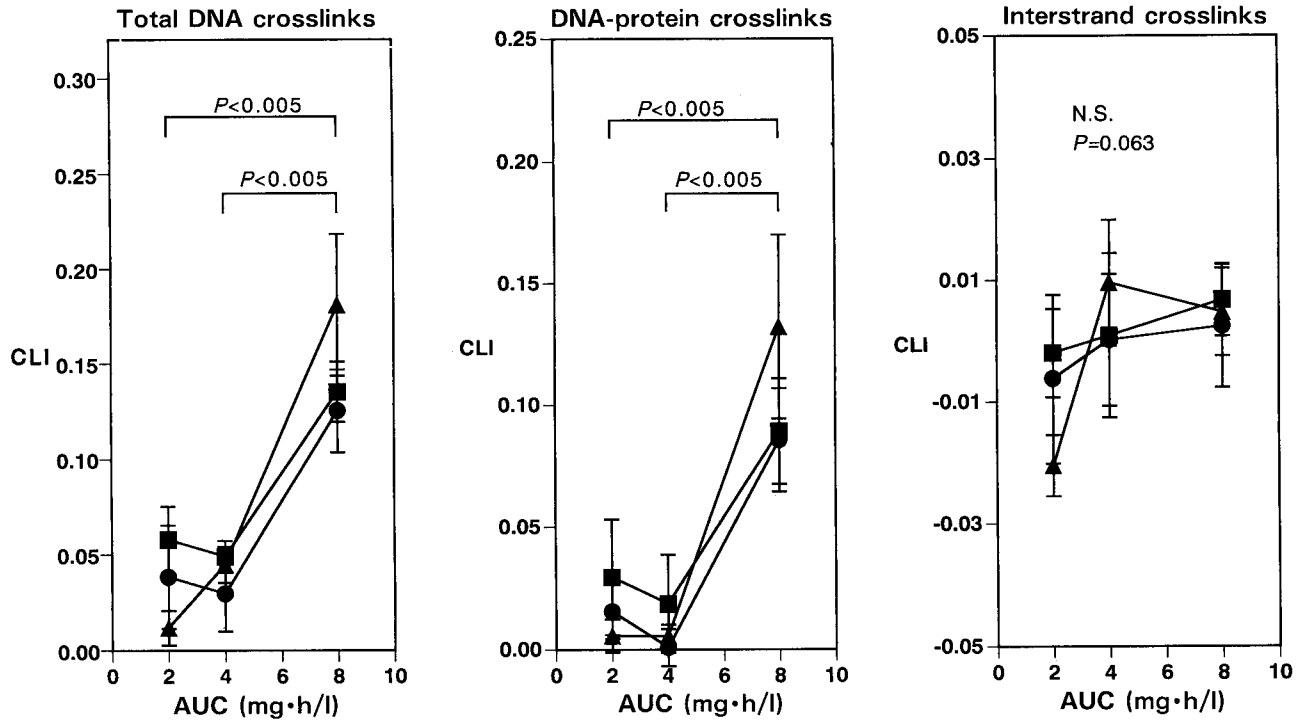

Fig. 5 The kinetics of crosslink formation. The crosslink index (CLI) of total DNA crosslinking (left), of DNA-protein crosslinking (center), and of DNA-DNA crosslinking (right) as a function of the AUC value is plotted. There were no differences in terms of drug exposure procedures as analyzed by the one-way analysis of variance test. This same test showed that AUC value is positively correlated with crosslink formation. The maximum AUC value in the clinical regimen, 8 mg·h/l, shows significantly more crosslink formation in the total number of DNA crosslinks and DNA-protein crosslinks by post hoc tests. (■: 4 mg/l; ●: 0.67 mg/l; ▲: Simulated *in vivo*)

Discussion

We measured and evaluated cell survival and DNA crosslink formation in a rat adenocarcinoma line in response to different concentrations of CDDP administered with varying exposure times. In doing so, we hoped to simulate the *in vivo* pharmacodynamics of CDDP in an *in vitro* setting. Since the simplest model of pharmacodynamics *in vitro* is considered to be a function of both drug concentration and drug exposure time, the simulated *in vivo* exposure method *in vitro* is considered to be an adequate experimental procedure to mimic *in vivo* phenomena *in vitro*. Moreover, because of the time-dependent nature of the cell membrane to various drugs and travel time from the membrane to the drug's target, it is possible that the drug exposure methods employing either a constant concentration or varying concentrations may not have the same pharmacodynamic effects even though the AUC values are identical. Ozawa *et al.* made a mathematical model of pharmacokinetic-pharmacodynamics for cell-cycle non-specific anti-cancer agents such as CDDP (1). The only pharmacodynamic aspect of the drug evaluated in their study was the surviving fraction of cells. Moreover, this model can only be used for drugs that can permeate the cell membrane and react with targets very quickly. Moreover, no study has attempted to mimic the *in vivo* pharmacokinetics of unbound CDDP. Therefore, it is useful to test the predictive accuracy of this model by simulating the *in vivo* CDDP concentration *in vitro* and to compare the results with the results of constant concentration exposure.

As for cell survival (evaluated by colony formation assay), the two kinds of constant drug concentration exposure yielded almost the same results statistically as the simulated exposure on the AUC bases as shown in Fig 2. Therefore, no matter how the CDDP exposure methods differ, cell survival is likely to be a function of the AUC value within the clinical dosage range. This result is identical to published data (3), despite the fact that our study took into account the CDDP reaction. This outcome supported the mathematical model. Because CDDP can kill cells by causing the formation of DNA crosslinks, the formation of DNA crosslinks was investigated as one of the important pharmacodynamic effects of CDDP. All of the alkaline elution assays in this study were performed at 4 h after the rinsing of CDDP as shown in Fig. 3, because the highest number of crosslinks were formed at this time as previously reported (7–9). Cell survival showed a strong correlation with the crosslink index of total DNA crosslinks. The logarithm of the cell survival was a linear function of the crosslink index ($R^2 = 0.85$). Therefore, the crosslink index of total DNA crosslinking is a good parameter to predict cell survival statistically.

DNA crosslinks caused by CDDP are classified as DNA-DNA crosslinks and DNA-protein crosslinks. In this study, the crosslink index, including both DNA-protein crosslinks and DNA interstrand crosslinks, showed a strong correlation with the survival of cells (Fig. 4) and with the AUC values statistically. The majority of the crosslinks were derived from DNA-protein crosslinks as shown in Fig. 5. Therefore, the results of this study indicate that DNA-protein crosslinking plays an important role in cytotoxicity within the clinical dosage range of CDDP. However, because we did not perform functional or metabolic analyses of the involved proteins in this study, the qualitative significance of DNA-protein crosslinking remains unclear. At the present time, we are performing a qualitative analysis of the role of DNA-protein crosslinking in cell survival. Zwelling *et al.* reported that since CDDP and *trans*-diammine-dichloroplatinum(II) (which induces about twice the amount of DNA-protein crosslinking as CDDP) yielded similar effects in terms of cell survival and interstrand DNA crosslink formation (6), it is thought that DNA-protein crosslinks are less important as lethal lesions. The high-mobility group (HMG) proteins that bind specifically to DNA crosslinks have been investigated recently. However, Cryer *et al.* reported that the HMG proteins 1 and 2, which display high affinity binding to CDDP-DNA crosslinks, can be removed from the CDDP-DNA crosslinks leaving the crosslink intact and capable of rebinding to HMG proteins (10). On the other hand, DNA-DNA crosslinks are classified as interstrand and intrastrand crosslinks. It is believed that cytotoxicity results from the formation of DNA interstrand crosslinks (11–16). Lemaire *et al.* reported that DNA interstrand crosslinks may be involved in the inhibition of transcription (17). Lawley and Phillips (18) reported that the mean lethal dose on the order of 100 interstrand crosslinks (19) (formed between CDDP and the N7 of two guanine residues at d (GC/GC) sites (17, 20)) were clearly the same magnitude as one found in cells cultured with mustard gas (21) which causes interstrand crosslinks. Hospers *et al.* reported that the amount of interstrand

crosslinks caused by CDDP showed a reverse correlation with CDDP resistance using cell lines (22). The general conclusion with regard to DNA crosslinks is that the platinum drugs broadly resemble mustard gas, with interstrand crosslinking being implicated as the most effective lethal lesion reported by Kohn *et al.* (11). Plooy *et al.* demonstrated that interstrand crosslinks and DNA-protein crosslinks required a longer time to repair (12), unlike intrastrand crosslinks. On the other hand, Pérez *et al.* reported that interstrand crosslinks caused by CDDP could be rearranged into intrastrand crosslinks on oligodeoxyribonucleotides (23). Therefore, the amount of CDDP-induced interstrand crosslinks might provide a crude estimate of total number of DNA-DNA crosslinks. Therefore, because the amount of DNA-protein crosslinks might be proportional to the amount of DNA-DNA crosslinks, it is likely that the strong correlation between cytotoxicity and the crosslink index of both DNA-protein crosslinks and interstrand crosslinks observed in this study can be explained.

Because cell survival was dependent on the AUC value of CDDP and because the crosslink index of DNA-protein crosslinks and the crosslink index of DNA interstrand crosslinks were strongly correlated with the AUC values, the method of drug administration in clinical practice is of great importance. The AUC value differed depending on the drug exposure time despite constant dose administration in a clinical study (5), which indicates that CDDP acts as a non-linear agent. Therefore, the results of this study indicate that not only dose intensity (mg/m^2/week), but also the AUC intensity (the AUC value per week: mg·h/l/m^2/week), should be considered when planning future chemotherapy regimens. To obtain higher AUC values, it is thought that consecutive low-dose administration yields the best outcome (24-27). When the AUC value is the same, low-dose consecutive CDDP administration might be superior to high-dose bolus administration because low-dose administration produces fewer side effects on patients. The threshold of DNA crosslinking and the AUC value should also be considered when planning chemotherapy regimens. In order to investigate the anti-tumor effects of unbound CDDP, we simulated *in vitro* the *in vivo* pharmacokinetics of CDDP with special attention to cell survival and DNA damage. The crosslink index of both DNA-protein crosslinks and interstrand crosslinks gave a quantitative indication of the degree of cytotoxicity. Our findings suggest that the optimum results of CDDP administration can be gained by aiming for a higher AUC intensity.

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References

1. Ozawa S, Sugiyama Y, Mitsuhashi Y, Kobayashi T and Inaba M: Cell killing action of cell cycle phase-non-specific antitumor agents is dependent on concentration-time product. Cancer Chemother Pharmacol (1988) **21**, 185-190.
2. Vermorken JB, van der Vijgh WJ, Klein I, Gall HE, van Groeningen CJ, Hart GA and Pinedo HM: Pharmacokinetics of free and total platinum species after rapid and prolonged infusions of cisplatin. Clin Pharmacol Ther (1986) **39**, 136-144.
3. Ma J, Verweij J, Kolker HJ, van Ingen HE, Stoter G and Schellens JH: Pharmacokinetic-dynamic relationship of cisplatin *in vitro*: Simulation of an i.v. bolus and 3h and 20h infusion. Br J Cancer (1994) **69**, 858-862.
4. Miyagi Y, Zhang H and Wheeler K: Radiation-induced DNA damage in tumors and normal tissues: IV. Influence of proliferation status and cell type on the formation of oxygen-dependent DNA damage in cultured cells. Radiat Res (1997) **148**, 29-34.
5. Belliveau JF, Posner MR, Ferrari L, Crabtree GW, Cummings FJ, Wiemann MC, O'Leary GP Jr, Griffin H, Phaneuf MA, O'Rourke A and Calabresi P: Cisplatin administration as a countinuous 5-day infusion: Plasma platinum levels and urine platinum excretion. Cancer Treat Rep (1986) **70**, 1215-1217.
6. Zwelling LA, Anderson T and Kohn KW: DNA-protein and DNA interstrand cross-linking by *cis*- and *trans*- platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxocity. Cancer Res (1979) **39**, 365-369.
7. Plooy AC, van Dijk M and Lohman PH: Induction and repair of DNA cross-links in chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxocity, mutagenicity, and antitumor activity. Cancer Res (1984) **44**, 2043-2051.
8. Strandberg MC, Bresnick E and Eastman A: The significance of DNA crosslinking to *cis*-diamminedichloroplatinum(II)-induced cytotoxocity in sensitive and resistant lines of murine leukemia L1210 cells. Chem-Biol Interactions (1982) **39**, 169-180.
9. Hettinga JV, Lemstra W, Meijer C, Dam WA, Uges DR, Konings AW, De Vries EG and Kampinga HH: Mechanism of hyperthermic potentiation of cisplatin action in cisplatin-sensitive and -resistant tumour cells. Br J Cancer (1997) **75**, 1735-1743.
10. Cryer JE, Johnson SW, Engelsberg BN and Billings PC: Analysis of HMG protein binding to DNA modified with the anticancer cisplatin. Cancer Chemother Pharmacol (1996) **38**, 163-168.
11. Kohn KW and Cheng YC: Development of Target-Oriented Anticancer Drugs. Raven Press, New York (1983) pp 181-188.
12. Plooy AC, van Dijk M, Berends F and Lohman PH: Formation and repair of DNA interstrand cross-links in relation to cytotoxocity and unscheduled DNA synthesis induced in control and mutant human cells treated with *cis*-diamminedichloroplatinum(II). Cancer Res (1985) **45**, 4178-4184.
13. Roberts JJ, Knox RJ, Pera MF, Friedlos F and Lydall PA: Platinum

and other metal coordination compounds; in Cancer Chemotherapy, Nicolini M ed, Nijhoff, Boston (1988) pp 16-31.
14. Roberts JJ, Chagas C and Pullman B: Molecular Mechanisms of Carcinogenic and Antitumor Activity. Pontifica Academia Scientarium, Vatican City (1987) pp 463-489.
15. Tomasz M, Lipman R, Crowdary D, Pawlak J, Verdine GL and Nakanishi K: Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science (1987) **235**, 1204-1208.
16. Zou Y, Van Houten B and Farrell N: Sequence specificity of DNA-DNA interstrand cross-link formation by cisplatin and dinuclear platinum complexes. Biochemistry (1994) **33**, 5404-5410.
17. Lemaire MA, Schwartz A, Rahmouni AR and Leng M: Interstrand cross-links are preferentially formed at the d(GC) sites in the reaction between *cis*-diamminedichloroplatinum(II) and DNA. Proc Natl Acad Sci USA (1991) **88**,1982-1982.
18. Lawley PD and Phillips DH: DNA adducts from chemotherapeutic agents. Mutat Res (1996) **355**, 13-40.
19. Roberts JJ and Friedlos F: The frequency of interstrand cross-links in DNA following reaction of *cis*-dimminedichloroplatinum(II) with cells in culture or DNA *in vitro*: Stability of DNA cross-links and their repair. Chem Biol Interact (1982) **39**, 181-189.
20. Huang H, Zhu L, Reid BR, Drobny GP and Hopkins PB: Solution structure of a cisplatin-induced DNA interstrand cross-link. Science (1995) **270**, 1842-1845.
21. Roberts JJ, Friedlos F, Scott D, Ormerod MG and Rawlings CJ: The unique sensitivity of Walker rat tumour cells to difunctional agents is associated with a failure to recover from inhibition of DNA systhesis and increased chromosome damage. Mutat Res (1986) **166**, 169-181.
22. Hospers GA, Meiler C, de Leij L, Uges DR, Mulder NH and de Vries EG: A study of human small-cell lung carcinoma (hSCLC) cell lines with different sensitivities to detect relevant mechanisms of cisplatin (CDDP) resistance. Int J Cancer (1990) **46**, 138-144.
23. Pérez C, Leng M and Malinge JM: Rearrangement of interstrand cross-links into intrastrand cross-links in *cis*-diamminedichloroplatinum(II)-modified DNA. Nucleic Acids Res (1997) **25**, 896-903.
24. Kaneta T, Takai Y, Nemoto K, Kakuto Y, Ogawa Y, Ariga H, Maruoka S, Ishibashi T, Hosoi Y and Yamada S: Effects of combination chemoradiotherapy with daily low-dose CDDP for esophageal cancer—results of a randomized trial. Jpn J Cancer Chemother (1997) **24**, 2099-2104 (in Japanese).
25. Saito Y, Mori K, Yokoi K, Tominaga K and Miyazawa N: Pilot phase II study of 5-day continuous infusion of cisplatin in treatment of non-small cell lung cancer. Jpn J Cancer Chemother (1989) **16**, 2081-2086 (in Japanese).
26. Terashima M, Ikeda K, Takagane A, Sasaki N, Abe K, Nishizuka S, Yonezawa H, Irinoda T, Nakaya T, Oyama K and Saito K: Pharmacokinetic analysis of low-dose intra-peritoneal *cis*-platinum administration. Jap J Cancer Chemother (1998) **25**, 1433-1435 (in Japanese).
27. Yagihashi A, Sasaki K, Hirata K and Yamamitsu S: Study of serum CDDP concentrations in patients with advanced or recurrent adeno-or squamous cell carcinoma under combination chemotherapy of 5-FU (CIV) and low-dose CDDP (IV). Jpn J Cancer Chemother (1996) **23**, 63-67 (in Japanese).

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