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Synergistic Enhancement of Cisplatin Cytotoxicity by SN-38, an Active Metabolite of CPT-11, for Cisplatin-resistant HeLa Cells

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A cisplatin (*cis*-diamminedichloroplatinum(II); CDDP)-resistant HeLa cell line (HeLa/CDDP cells), which showed more than 8-fold resistance to CDDP compared to the parent cells, was newly established for this study. HeLa/CDDP cells accumulated 50% less platinum than the parent cells. There was no difference in intracellular glutathione (GSH) content between the parent and HeLa/CDDP cells. The dose modification factor by DL-buthionine-S,R-sulfoximine (BSO) pretreatment was similar in both cell lines. HeLa/CDDP cells had cross-resistance to diammine(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA), (*cis*-diammine(glycolato)platinum (254-S), but not to (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)platinum(II) (DWA2114R), adriamycin, or VP-16. HeLa/CDDP cells showed a collateral sensitivity to 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11). Furthermore, isobologram analysis indicated synergistic interaction of CDDP and SN-38 only for HeLa/CDDP cells. The present study suggests that combination therapy with CDDP and CPT-11 may be potentially useful in the treatment of some patients with CDDP-resistant cancer.

Key words: CDDP-resistance — CPT-11 — HeLa cell

Cisplatin (*cis*-diamminedichloroplatinum(II); CDDP) is the most active anticancer agent and has broad clinical application.[1] However, intrinsic or acquired resistance to the cytotoxic effect of this useful agent frequently leads to chemotherapeutic failure.[2] A number of cell lines with acquired resistance to CDDP have been developed (for reviews, see Scanlon *et al.*,[3] Andrews and Howell[4]), and those cell lines have provided model systems for the studies on the mechanisms for CDDP resistance. Several mechanisms of cellular resistance to CDDP have been proposed, such as decreased drug cellular accumulation, enhanced inactivation of the drug by the intracellular detoxication system, decreased DNA damage and/or increased repair.[3,4] We have examined the resistance mechanism in CDDP-resistant HeLa cells newly established in our laboratory for this study.

The combination of CDDP and another anticancer agent seems to be essential in clinical practice, although several approaches for overcoming the resistance have been tried. Experimental study on the combined effect of CDDP and other cytotoxic agents can provide information on the most effective sequence of treatment modalities. Recently, much attention has been paid to DNA topoisomerases as therapeutic targets in cancer chemotherapy.[5] Epipodophyllotoxin VP-16, a DNA topoisomerase II inhibitor, has been widely used in clinical practice.[6,7] Camptothecin inhibits DNA topoisomerase I through the formation of stable topoisomerase I-DNA cleavable complexes.[8,9] CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, a semisynthetic water-soluble derivative of camptothecin, has a potent antitumor activity.[10,11] SN-38, 7-ethyl-10-hydroxycamptothecin, an active metabolite of CPT-11, also has a strong inhibitory activity against topoisomerase I *in vitro*.[12,13] Although several authors have focused on the combined effect of CDDP and CPT-11,[14,15] the combined effect of those agents on CDDP-resistant cells has not been elucidated.

Therefore, in this study we focused on the combined effects of CDDP with VP-16 or SN-38 on HeLa and CDDP-resistant HeLa cells.

MATERIALS AND METHODS

Cell line and culture Experiments were conducted using HeLa cells and a CDDP-resistant subline (HeLa/CDDP cells). HeLa/CDDP cells were established by continuous exposure of HeLa cells to stepwise escalating concentrations of CDDP for 6 months. HeLa/CDDP cells showed more than 8-fold resistance to CDDP compared to the parent cells. Both cell lines were maintained in Eagle's minimum essential medium (Nissui Seiyaku, Tokyo) containing 2 m*M* glutamine, 100 IU/ml of penicillin, and 10% fetal bovine serum (Bocknek, Canada) at 37°C in a humidified incubator with 95% air and 5% CO_2. Before

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each experiment, HeLa/CDDP cells were cultured with drug-free medium for 3 weeks.

Drug and chemicals The drugs were obtained from the following sources: CDDP and VP-16, Nippon Kayaku, Tokyo; diammine(1,1-cyclobutanedicarboxylato)platinum (CBDCA), Bristol-Myers Squibb, Princeton, NJ; Adriamycin (ADM), Kyowa Hakko, Tokyo; *cis*-diammine(glycolato)platinum (254-S), Shionogi, Osaka; (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)platinum(II) (DWA2114R), Chugai, Tokyo; SN-38, Yakult, Tokyo. DL-Buthionine-S,R-sulfoximine (BSO) and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Platinum accumulation HeLa and HeLa/CDDP cells in late-logarithmic phase were harvested and seeded into tissue culture flasks at the density of 2×10^6 cells/ml. Twenty-four hours after incubation, they were exposed to 15 μM CDDP in Eagle's minimum essential medium supplemented with 10% fetal bovine serum for 2, 4 or 8 h. The cell extracts were analyzed for platinum by using a flameless atomic absorption spectrometer AA275 (Varian, Australia) according to LeRoy's method.[16] Four independent experiments were performed for each exposure period.

GSH content and BSO treatment The stock cell pellets were sonicated using a ultrasonic disrupter Model 200 (Tomy Seiko, Tokyo). The total glutathione (GSH) contents of the cell lines were determined by an enzymatic assay utilizing GSH reductase according to Griffith's method.[17] Cells (5×10^5) were plated in tissue culture flasks with growth medium and incubated for 48 h. Then, to deplete the GSH in the cells, BSO (a γ-glutamylcysteine synthetase inhibitor) was added to the flasks. The final concentration of BSO was 50 μM.[18] After 24 h, cells were harvested and used for MTT assay. The degree of sensitization induced by BSO treatment was expressed as a mean dose modification factor, defined as IC_{50} to CDDP of the control cells/IC_{50} of GSH-depleted cells. This BSO exposure produced no cytotoxicity in either parent or resistant cells. For the studies on GSH content and the mean dose modification factor, HeLa/CDDP cells were divided into two subgroups. Cells of one group were cultured with drug-free medium for 3 weeks before experiments as usual (HeLa/CDDP). Cells of the other group were continuously exposed to 3.0 μM CDDP, which was added for this experiment (HeLa/c-CDDP). Protein content in the cells was assayed according to the method of Lowry *et al.*[19]

Sensitivity to anticancer agents and combination assay The MTT assay system used was essentially the same as that reported by Mosmann.[20] Briefly, cells were diluted with culture medium to the seeding density, suspended in 96-well tissue culture plates (120 μl/well) (Sumitomo Bakelite, Tokyo), and preincubated at 37°C for 4 h. Cells were then treated continuously with 30 μl of various concentrations of the anticancer agents to obtain a dose-response curve for each agent. For combination assay, cells were also treated continuously with 15 μl of various concentrations of CDDP and VP-16 or SN-38. Seeding cell number (10^4 cells/well) and incubation time (96 h) were determined from the linear relationship between the absorbance and number of cells in the growth curve of each cell line. Drug concentrations were as follows; 0.1–20 μM CDDP, 1.0–200 μM CBDCA, 0.1–20 μM 254-S, 0.1–100 μM DWA2114-R, 0.01–100 μM ADM, 0.01–100 μM VP-16, 0.1–100 nM SN-38. Each plate had one control column (8 wells) containing medium without cells and another one including cells without drugs. After incubation for 92 h, 20 μl of MTT solution (2.5 mg/ml) was added to each well and the plates were further incubated for 4 h. One hundred and fifty μl of dimethyl sulfoxide was added and the plates were vigorously shaken on a plate-shaker to solubilize the MTT-formazan product. Absorbance at 570 nm was measured with a microplate reader Model 450 (BIO-RAD, Richmond, CA).

Dose-response curves were plotted on a semi-log scale as percentage of the control cell number, which was obtained from the no-drug-exposure sample. The resistance factor of each anticancer agent was defined as IC_{50}

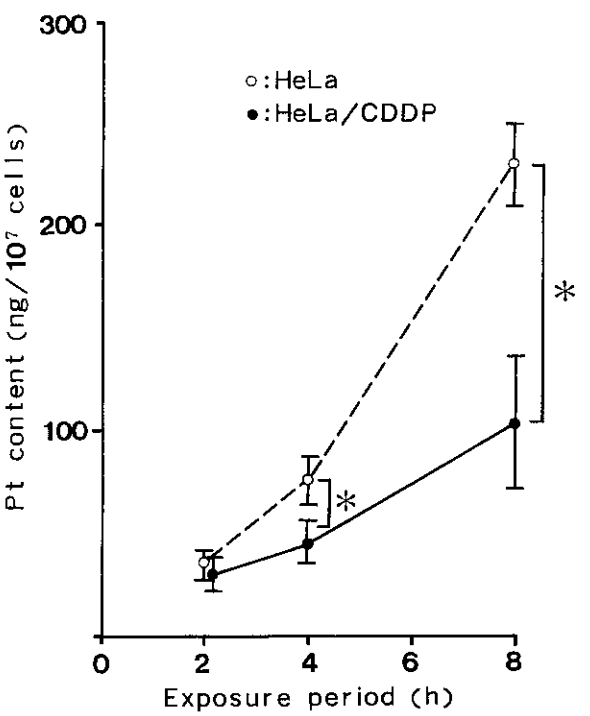

Fig. 1. Platinum accumulation (mean±SD) in HeLa and HeLa/CDDP cells after CDDP exposure (* $P<0.05$).

of HeLa/CDDP cells/IC_{50} of HeLa cells. The effects of CDDP in combination with SN-38 or VP-16 on HeLa and HeLa/CDDP cells at the IC_{50} were analyzed by using an improved isobologram method.[14, 21]

RESULTS

Platinum accumulation Up to 2 h after exposure to CDDP, there was no significant difference in platinum accumulation between HeLa and HeLa/CDDP cells. HeLa/CDDP cells accumulated 50% less platinum than the parent cells at 8 h (Fig. 1).

GSH content and BSO treatment GSH content (mean ± SD μg/mg protein) was 8.08±2.26 for HeLa cells, 8.36±2.84 for HeLa/CDDP cells, and 30.2±6.42 for HeLa/c-CDDP. There was a significant difference in GSH content between HeLa/c-CDDP and HeLa or HeLa/CDDP cells ($P<0.05$). The mean dose modification factor by BSO treatment was 1.4 for the parent cells, 1.1 for HeLa/CDDP cells, and 1.2 for HeLa/c-CDDP.

Sensitivity to anticancer agents and combination assay HeLa/CDDP cells showed cross resistance to CBDCA and 254-S, but not to DWA2114R, ADM, or VP-16. They showed a collateral sensitivity to SN-38 (Table I).

Table I. Cytotoxicity of Each Agent to HeLa and HeLa/CDDP Cells

Agent	IC_{50} (μM)		Resistance factor
	HeLa	HeLa/CDDP	
CDDP	1.1	9.0	8.2
CBDCA	13.0	140.0	10.7
254-S	7.2	50.0	6.9
DWA2114R	16.0	20.0	1.3
ADM	0.8	0.4	0.5
VP-16	1.9	2.0	1.1
SN-38	3.8×10^{-2}	0.8×10^{-2}	0.2

The resistance factor is the IC_{50} of HeLa/CDDP cells divided by the IC_{50} of HeLa cells.

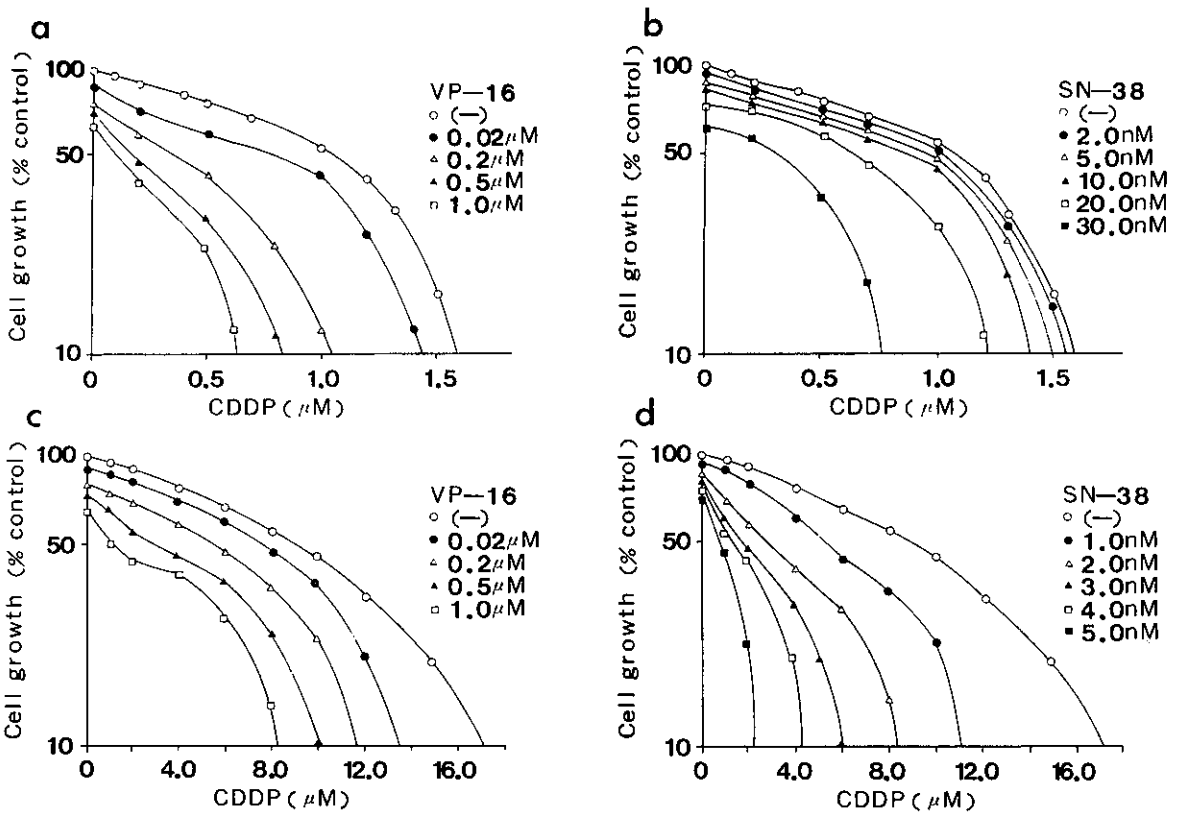

Fig. 2. Dose-response curves of HeLa cells for CDDP in combination with VP-16 (a) and SN-38 (b), and those of HeLa/CDDP cells for CDDP in combination with VP-16 (c) and SN-38 (d). The concentrations of VP-16 and SN-38 for each symbol are shown on the right. CDDP concentrations are shown on the abscissa. Each assay was run in triplicate, and cell growth number was plotted as a percentage of the control (cells not exposed to drugs).

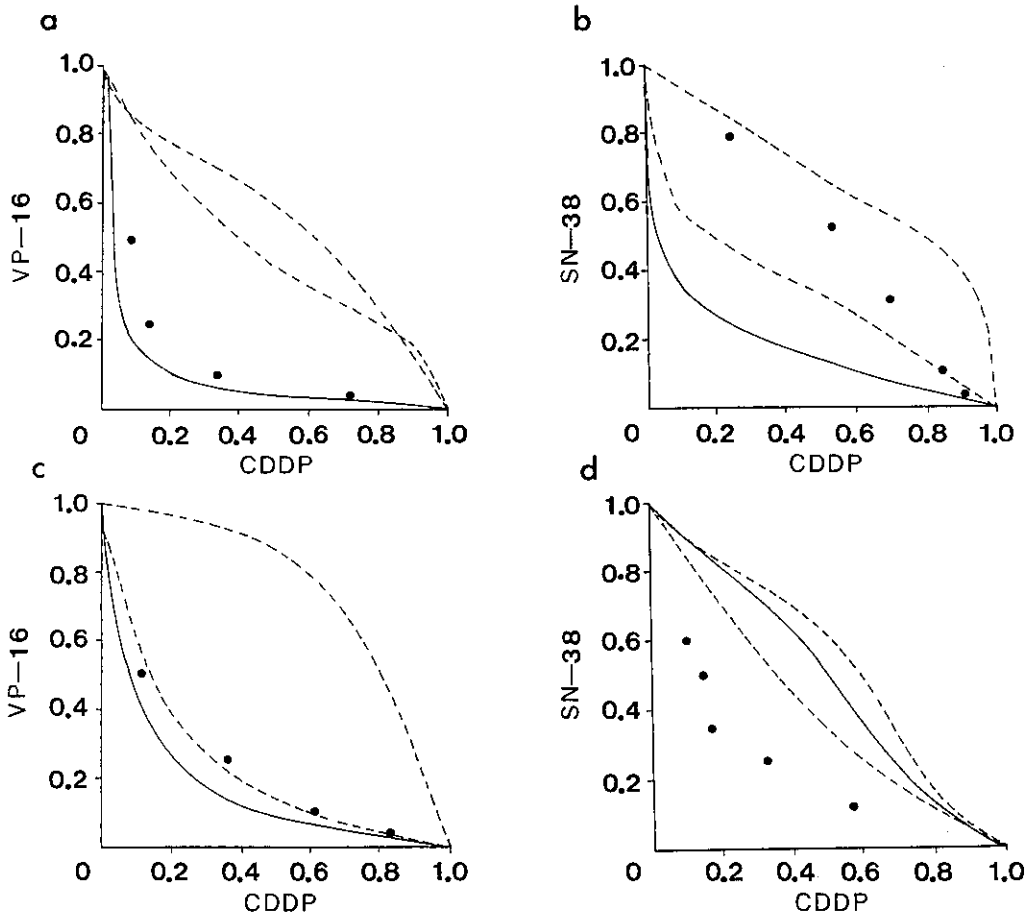

Fig. 3. Isobolograms of CDDP at IC_{50}, based on dose-response curves for the combination with VP-16 (a) or SN-38 (b) for HeLa cells, and those for the combination with VP-16 (c) or SN-38 (d) for HeLa/CDDP cells. The abscissa shows the concentration ratio to IC_{50} for CDDP, and the ordinate represents that for VP-16 or SN-38. A solid line shows the mode I line and the two broken lines show the mode II lines.[14, 21]

Dose-response curves of CDDP in combination with VP-16 or SN-38 for both cell lines are shown in Fig. 2. Isobolograms at IC_{50} based upon these dose-response curves in combination were prepared (Fig. 3). For the exposure to CDDP and VP-16, the combined data points fell within the envelope surrounded by the three lines in each cell line (Fig. 3a, c). This observation was interpreted as showing that the exposure to CDDP and VP-16 produced only an additive effect on each cell line. For the combination of CDDP with SN-38, the data points fell within the envelope of additivity in HeLa cells (Fig. 3b). On the other hand, the data points of the combination of CDDP with SN-38 reached the left of the envelope for HeLa/CDDP cells (Fig. 3d). This combination effect on HeLa/CDDP cells was regarded as supra-additive (synergistic).

DISCUSSION

HeLa/CDDP cells showed reduced intracellular platinum accumulation compared to the parent cells. This phenomenon has been reported in other CDDP-resistant human tumor cell lines.[4] The precise mechanism of the reduction has not been elucidated, although a number of factors may influence the intracellular platinum content such as the balance between influx and efflux, reversible protein binding, intracellular compartmentalization, binding of the ultimate electrophile to a cellular target, and removal of the ultimate damage.[4]

There was no difference in intracellular GSH contents between the parent and HeLa/CDDP cells. However, the dose modification factor by BSO treatment did not differ among these cell lines. Accordingly, the intracellular

detoxication system involving GSH-related enzymes may not play an important role in the resistance mechanism in this cell line.

As compared to the parent cells, the more than 8-fold resistance to CDDP in HeLa/CDDP cells could not be simply explained by the reduced platinum accumulation. The mechanism of DNA repair in mammalian cells and the mode of repair of CDDP-induced cross-links are not yet clear. Therefore, it is difficult to discuss the enzymatic repair mechanisms in relation to drug resistance, although a previous study on another CDDP-resistant HeLa cell line which was selected by intermittent exposure to CDDP, showed DNA repair as a potential resistance mechanism.[22]

HeLa/CDDP cells showed no cross-resistance to ADM. Enhanced mdr1 mRNA was not detected in this resistant cell line (data not shown). Therefore, it is not likely that the resistance to CDDP in this cell line involves a similar mechanism to that in multidrug-resistant cells.[23] HeLa/CDDP cells showed cross resistance to CBDCA and 254-S, but not to DWA2114R. Misawa *et al.* reported that a CDDP-resistant ovarian cancer cell line showed less cross-resistance to DWA2114R and platinum accumulation in the resistant cells showed the lowest reduction for DWA2114R among platinum compounds.[24]

DNA topoisomerases are enzymes which regulate the superhelical density of DNA by transiently nicking either one (type I) or both (type II) strands of the DNA helix. Topoisomerases may be involved in many aspects of DNA metabolism, including transcription, replication, recombination, and chromosome segregation.[5] Synergistic enhancements of CDDP cytotoxicity by VP-16 have been reported.[6] But in the present study, the combination of CDDP and VP-16 showed additive effects without synergism in HeLa and HeLa/CDDP cells. They had such a high sensitivity to VP-16 that the isobolograms of the combination of CDDP and VP-16 showed large envelopes. This may be why synergism between CDDP and VP-16 could not be detected.

Tsuruo *et al.*[11] have demonstrated that CPT-11 is effective against pleiotropic drug-resistant tumors *in vitro* and *in vivo*, and Masuda *et al.*[25] reported that CPT-11 shows some lack of cross resistance with CDDP. There has been a previous experimental study demonstrating synergism between CDDP and SN-38,[14] and clinical studies have demonstrated that the CPT-11/CDDP combination is more efficacious than CPT-11 alone.[15] In this study, HeLa/CDDP cells showed a collateral sensitivity to SN-38, although a previous study reported cross resistance to SN-38 in a CDDP-resistant human ovarian cancer cell line.[26] Furthermore, for the combination of CDDP with SN-38, the isobologram demonstrated synergistic interaction for HeLa/CDDP cells, but not for the parent cells. This is the first study that has demonstrated synergism between CDDP and SN-38 in a CDDP-resistant cell line, although the mechanisms of this collateral sensitivity to SN-38 and of the synergism only for the CDDP-resistant cell line are unknown.

While further study is necessary, the present study suggests that combination therapy with CDDP and CPT-11 may be potentially useful in the treatment of some patients with CDDP-resistant cancer.

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