

## Induction of Cell Death by Combination Treatment with Cisplatin and 5-Fluorouracil in a Human Oral Squamous Cell Carcinoma Cell Line

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**Abstract.** *The possible apoptosis-inducing activity of several sequential treatments of cisplatin (CDDP) and 5-fluorouracil (5-FU) against the human oral squamous cell carcinoma HSC-2 cell line was investigated. The following three combination treatments (CT) were used: simultaneous treatment with CDDP and 5-FU (for 72 hours) (CT-1), CDDP treatment (24 hours) followed by 5-FU treatment (48 hours) (CT-2) and 5-FU treatment (24 hours) followed by CDDP treatment (48 hours) (CT-3). CT-1 produced the highest cytotoxicity, followed by CT-3 and CT-2. No treatment induced any detectable internucleosomal DNA fragmentation, and caspase-3,-8 and -9 were activated to a much lesser extent than that attained using actinomycin D. High-performance liquid chromatography analysis demonstrated that 5-FU, as well as CT-1 and CT-2, preferentially reduced the intracellular concentration of putrescine. These results suggest that simultaneous treatment with CDDP and 5-FU induces lower level of apoptotic cell death in HSC-2 cells.*

Cisplatin (CDDP) and 5-fluorouracil (5-FU) have been clinically used for the treatment of various malignant tumors, such as head and neck (1-7), gastric, esophageal, ovarian and lung cancer (8-11). The antitumor potency of combinational treatment with CDDP and 5-FU against various cancer cells has been well documented. However, very few studies have dealt with the development of an optimum treatment schedule of CDDP and 5-FU against head and neck cancer cells (12, 13). Therefore, here we

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investigated the possible apoptosis-inducing activity of three combination treatments with CDDP and 5-FU (CT-1 to 3). The apoptosis markers we adopted were caspase-3,-8 and -9 activation, and DNA fragmentation. Polyamines such as putrescine, spermidine and spermine have been reported to be involved in cell proliferation, survival and death. The elevation and decline of the intracellular polyamine concentration has been reported to be linked to malignant transformation and apoptosis induction, respectively (14). In accordance with this, we have found that the intracellular putrescine level declined during the early stage of apoptosis induced by etoposide, epigallocatechin gallate or ascorbates, whereas spermidine and spermine levels were almost unchanged (15, 16). We therefore investigated here the changes of polyamine levels induced by sequential treatments with CDDP and 5-FU in HSC-2 cells.

### Materials and Methods

**Materials.** The following chemicals and reagents were obtained from the companies indicated: Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem., St. Louis, LA USA); 5-FU (Kyowa, Tokyo, Japan); CDDP (Briplatin injection, Bristle Pharmaceutical Co., Tokyo, Japan). Other materials for high-performance liquid chromatography (HPLC) were obtained from Wako Pure Chem. Ind., Ltd. Osaka, Japan.

**Cell culture.** HSC-2 cells were cultured at 37°C in DMEM supplemented with 10% heat-inactivated FBS in the tissue culture dish (Falcon 100x20 mm Style) (Becton Dickinson Labware, NJ, USA), under a humidified 5% CO<sub>2</sub> atmosphere. Human promyelocytic leukemic HL-60 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS under a humidified 5% CO<sub>2</sub> atmosphere. HSC-2 and HL-60 cells were obtained from the Riken Cell Bank.

**Treatment combinations.** The following three combination treatments were adopted: (i) simultaneous treatment with CDDP and 5-FU for 72 hours (CT-1), (ii) CDDP treatment (24 hours) followed by 5-FU treatment (48 hours) (CT-2) and (iii) 5-FU

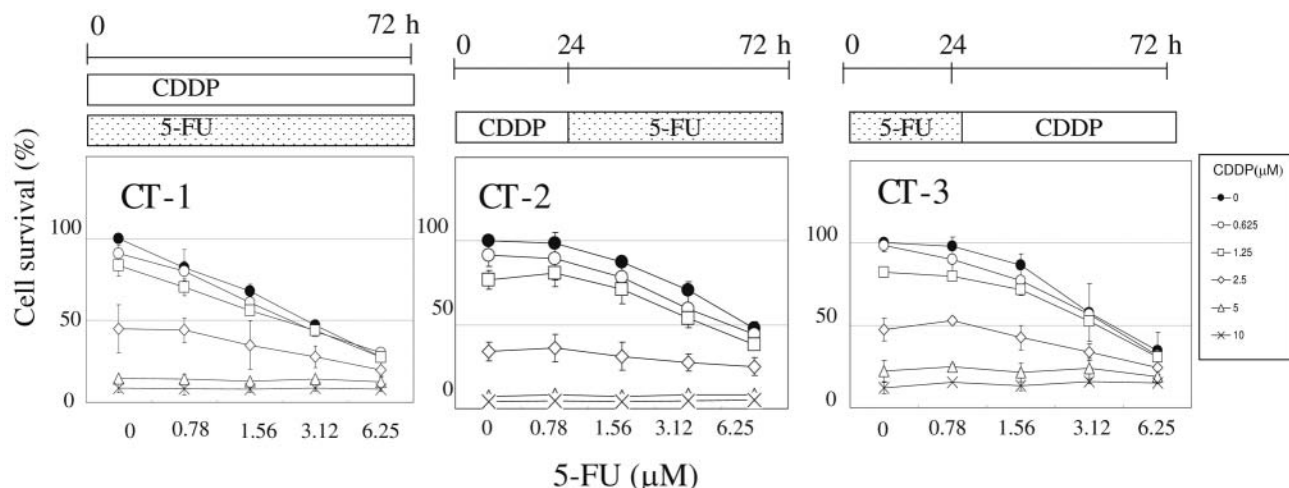


Figure 1. Cytotoxic activity of combination treatments with CDDP and 5-FU against HSC-2 cells. Near confluent HSC-2 cells were incubated for 72 hours with the indicated concentrations of CDDP or 5-FU alone, or in combination (CT-1), or sequentially (CT-2, CT-3) and the relative viable cell number was determined by MTT method. Each value represents mean  $\pm$  S.D. from 3 independent experiments.

treatment (24 hours) followed by CDDP treatment (48 hours) (CT-3). For the cytotoxic assay, HSC-2 cells ( $2 \times 10^3/0.1$  mL) were inoculated in 96-microwell plates (Becton Dickinson) and incubated for 24 hours before the combination treatments described above (CT-1 to 3). For the DNA fragmentation and polyamine assays, HSC-2 cells ( $4 \times 10^4/5$  mL) were inoculated to 6-well tissue culture plates (Becton Dickinson) and incubated for 24 hours before treatment. For the caspase assay, HSC-2 cells ( $2 \times 10^5/10$  mL) were inoculated to the tissue culture dish (Falcon) and treated for 24 hours before treatment. As a control treatment, medium alone was used.

**Assay for cytotoxic activity.** Cells were treated for 72 hours with various concentrations (0.625 to 10  $\mu$ M CDDP and 0.78 to 6.25  $\mu$ M 5-FU) of CDDP or 5-FU alone, or in combination treatments (CT-1 to 3) with three replicate wells for each concentration. The medium was replaced with 0.1 mL fresh medium containing various concentrations test compounds per 24 hours according to each combination treatment schedule (CT-1 to 3). The relative viable cell number of adherent cells was then determined by the MTT method. In brief, the drug-treated cells were washed once with phosphate-buffered saline (PBS) without  $Mg^{2+}$  and  $Ca^{2+}$  [PBS(-)], and incubated for 4 hours with 0.2 mg/mL MTT in the culture medium. After removal of the culture medium, cells were then lysed with 100  $\mu$ L of DMSO and the absorbance at 540 nm of the cell lysate was measured by a microplate reader (Labsystems Multiskan, Biochromatic Labssystem, Osaka, Japan) attached to a Star DOT Matrix printer JL-10. The 50% cytotoxic concentration ( $CC_{50}$ ) was determined from the dose-response curve (17).

**Assay for DNA fragmentation.** The pelleted HL-60 cells ( $5 \times 10^5$ /well) or HSC-2 cells (collected by scraping with a rubber policeman) were lysed, and digested with RNase A and proteinase K (Boehringer Ingelheim GmbH, Ingelheim, Germany). The DNA was then isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (17). The DNA from apoptotic cells induced by actinomycin D (Act-D) (1  $\mu$ g/mL, 6 hours treatment) was run in parallel as a positive control.

**Assay for caspase activation.** HSC-2 cells were washed twice with PBS(-) and lysed with lysis solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice and centrifugation for 10 minutes at 10,000  $g$ , the supernatant was collected. Lysate (50  $\mu$ L, equivalent to 100  $\mu$ g protein) was mixed with 50  $\mu$ L of 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-pNA (*p*-nitroanilide)), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA) (MBL, Nagoya, Japan). After incubation for 4 hours at 37°C, the absorbance at 405 nm of the liberated chromophore pNA was measured by a microplate reader. Comparison of the absorbance of pNA generated by the lysate of treated cells with that of a control (untreated) cell lysate allows the determination of the relative caspase activity (expressed as % of control), according to the manufacturer's instructions (MBL).

**Determination of polyamines.** HSC-2 cells were harvested by trypsinization, washed twice with PBS(-), and extracted with 10% trichloroacetic acid (TCA). After centrifugation for 5 minutes at 10,000 $g$ , the deproteinized supernatant was collected and stored at -40°C. The polyamines in the supernatant were determined by HPLC, after dansyl-derivatization. A modified procedure of dansylation was performed, according to a previously described method (18). Fifty  $\mu$ L of the supernatant (or of the standard solution), 10  $\mu$ L internal standard (IS; 1,6-diaminohexane-2HCl) solution, 50  $\mu$ L of saturated  $Na_2CO_3$  solution, 300  $\mu$ L of dansyl chloride (DNS) solution (3 mg/mL acetonitrile) and 400  $\mu$ L of extra-pure water were added. After being vortexed, the mixture was stood for 15 minutes at room temperature. The dansylated derivatives were extracted into 0.2 mL of toluene. The extract was evaporated in a stream of air, and the residue was dissolved in 200  $\mu$ L of 80% acetonitrile in water; 10  $\mu$ L was injected into the HPLC system. HPLC separation was performed with a SHISEIDO CAPCELL PAK  $C_{18}$  column (4.6 mm i.d., 35 mm in length). The mobile phase for elution was a linear gradient between 50% acetonitrile in water (mobile phase A) and 80% acetonitrile in water (mobile phase B), time program was described below (0-5 min, A 100%; 5-13 min, A 100-0%; 13-20 min, A 0%; 20-25 min, A 0-100%) at a flow rate of

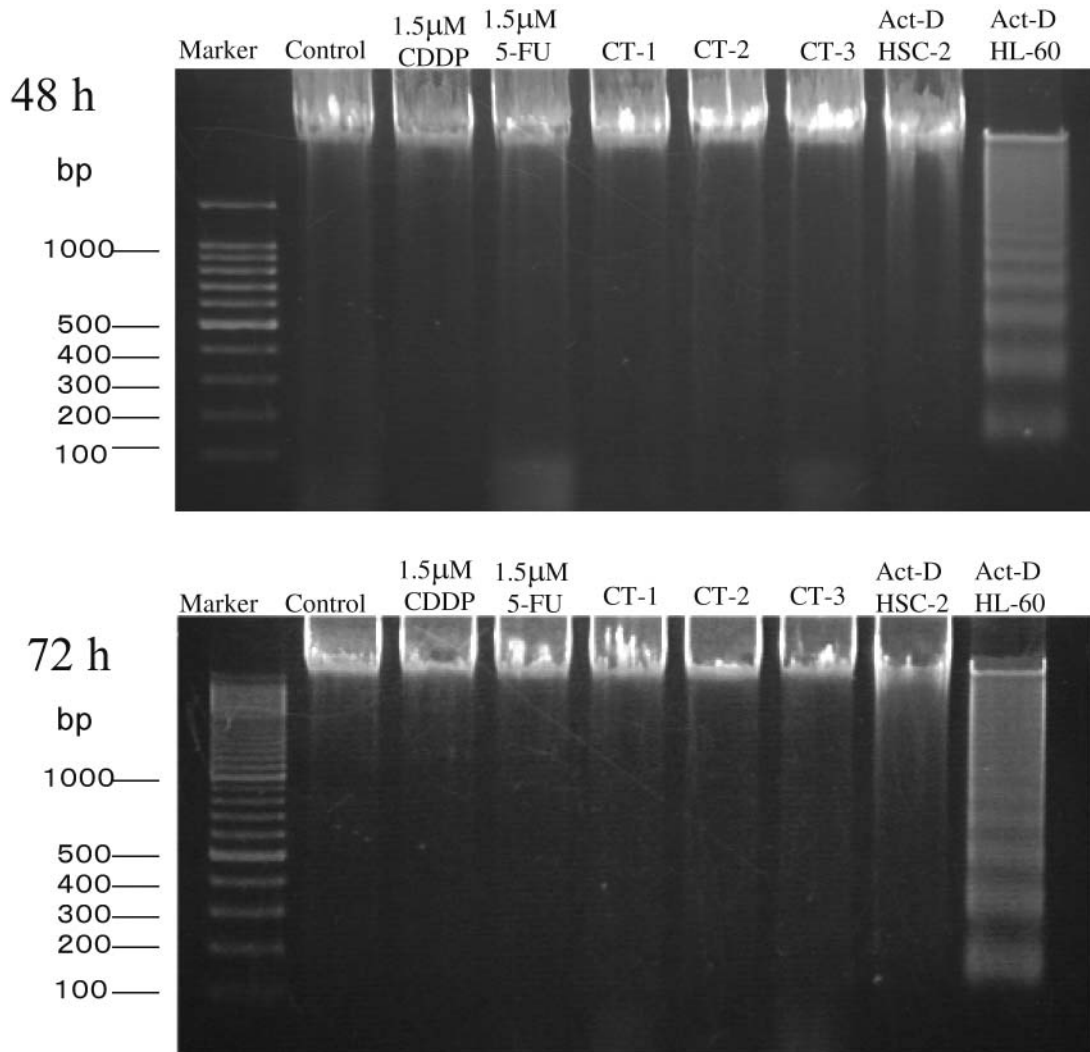


Figure 2. Induction of DNA fragmentation by combination treatment of HSC-2 cells with CDDP and 5-FU. Near confluent HSC-2 cells were incubated for 48 or 72 hours, alone (control), with 1.5  $\mu\text{M}$  CDDP or 1.5  $\mu\text{M}$  5-FU alone, or applied in combination (CT-1) or sequentially (CT-2, CT-3), or with actinomycin D (1  $\mu\text{g}/\text{mL}$ : 6 hours), and were then assayed for DNA fragmentation. Act-D HL-60, DNA from apoptotic HL-60 cells induced by actinomycin D (1  $\mu\text{g}/\text{mL}$ : 6 hours).

0.7 mL/min. Fluorescence intensity was measured using excitation at 337 nm and emission at 521 nm. The polyamine concentration was normalized by the viable cell number.

## Results

**Induction of cytotoxic activity by CDDP and 5-FU.** All sequential treatments of CDDP and 5-FU (CT-1 to 3) reduced the viable HSC-2 cell count in an additive fashion. In the absence of CDDP, the CT-1 treatment schedule induced the highest cytotoxicity in HSC-2 cells ( $\text{CC}_{50}$  of 5-FU =  $2.95 \pm 0.30 \mu\text{M}$ ), followed by CT-3 ( $5.26 \pm 0.30 \mu\text{M}$ ) and then CT-2 ( $5.93 \pm 0.60 \mu\text{M}$ ). When CDDP was added at the 0.625  $\mu\text{M}$ , CT-1 induced the highest cytotoxicity ( $\text{CC}_{50}$  of 5-

FU =  $2.51 \pm 0.20 \mu\text{M}$ ), followed by CT-3 ( $4.09 \pm 0.40 \mu\text{M}$ ) and CT-2 ( $4.67 \pm 1.30 \mu\text{M}$ ). When CDDP was added at 1.25  $\mu\text{M}$ , the CT-1 treatment schedule again induced the highest cytotoxic activity ( $\text{CC}_{50}$  of 5-FU =  $2.31 \pm 0.20 \mu\text{M}$ ), followed by CT-3 ( $3.48 \pm 0.20 \mu\text{M}$ ) and CT-2 ( $3.75 \pm 0.80 \mu\text{M}$ ). These data suggest that the CT-1 treatment schedule with CDDP and 5-FU induces the greatest cytotoxicity against HSC-2 cells (Figure 1).

**Lack of DNA fragmentation.** Neither CDDP (1.5  $\mu\text{M}$ ) nor 5-FU (1.5  $\mu\text{M}$ ) induced internucleosomal DNA fragmentation in HSC-2 cells. Furthermore, the combination treatments (CT-1 to 3) were also inactive,

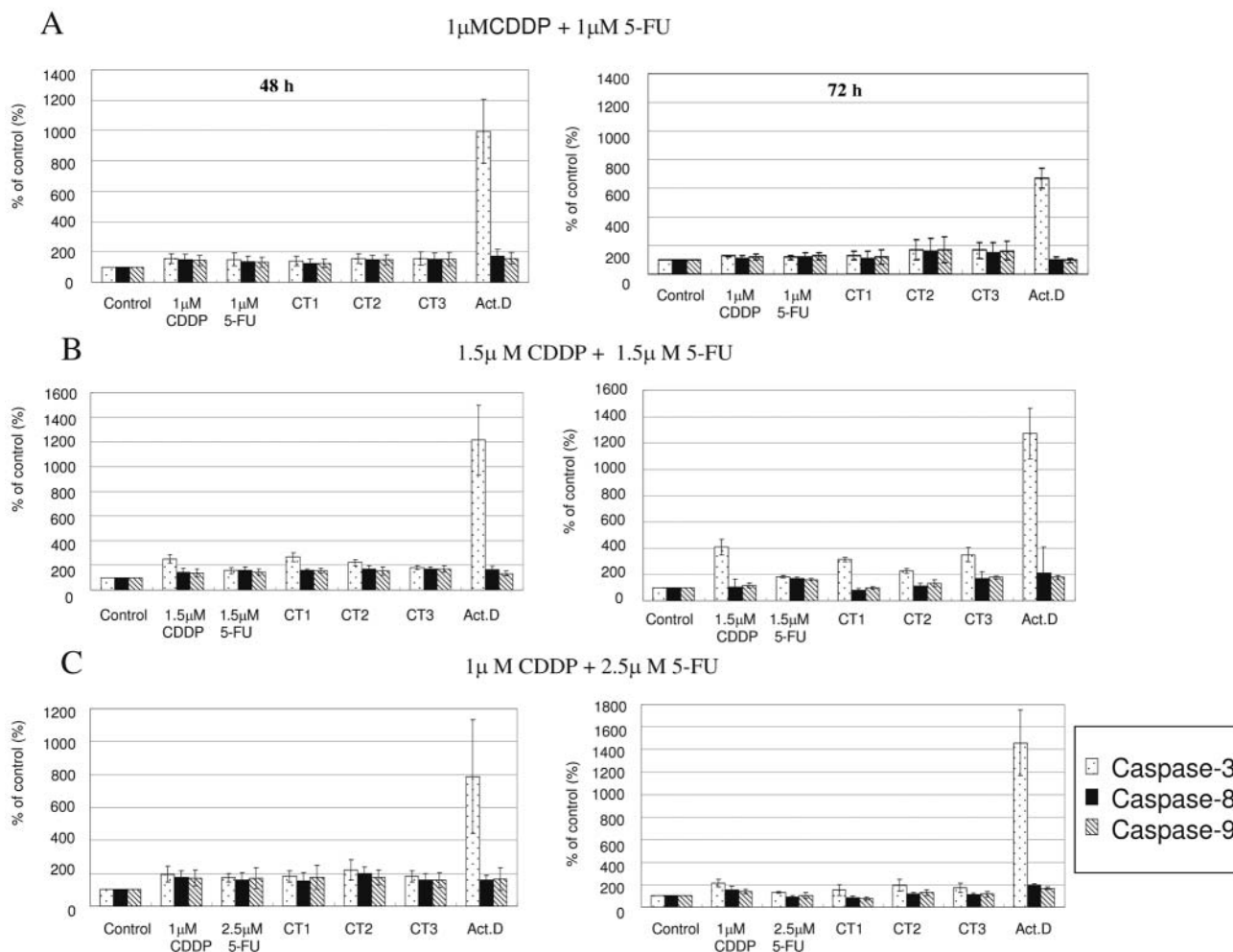


Figure 3. Activation of caspase-3, 8 and -9 by combination treatment of HSC-2 cells with CDDP and 5-FU. Near confluent HSC-2 cells were incubated for 48 or 72 hours with the indicated concentrations of CDDP or 5-FU alone, or applied in combination (CT-1) or sequentially (CT-2, CT-3), or with actinomycin D (1  $\mu\text{g}/\text{mL}$ ; 4 h). Caspase activity was assayed and expressed as a % that of the control. Each value represents mean  $\pm$  S.D. from 3 independent experiments.

regardless of the incubation time (either 48 or 72 hours) with HSC-2 cells (Figure 2). Even Act-D (1  $\mu\text{g}/\text{mL}$ , 6 hours) failed to induce internucleosomal DNA fragmentation in HSC-2 cells, although it did induce the laddering pattern of DNA fragmentation in HL-60 cells (Figure 2). The combination of CDDP (1  $\mu\text{M}$ ) and 5-FU (1  $\mu\text{M}$ , 2.5  $\mu\text{M}$ ) in combination treatments (CT-1 to 3) did not induce internucleosomal DNA fragmentation in HSC-2 cells regardless of the incubation time (either 48 or 72 hours) (data not shown). This suggests that HSC-2 cells are relatively resistant to the induction of DNA fragmentation, as compared with HL-60 cells.

*Low level of caspase activation.* CDDP alone dose- and time-dependently activated caspase-3 up to a level 4-fold

that of the control (1.5  $\mu\text{M}$ , 72 hours), but did not activate caspase-8 or -9. 5-FU alone activated caspase-3, -8 and -9, to lesser extent (at most, 2-fold). Combination treatments (CT-1 to 3) did not further activate the caspases (Figure 3).

*Determination of polyamines.* HPLC analysis demonstrates that treatment with 5-FU alone (1.5  $\mu\text{M}$ , 72 hours) resulted in the selective decline of the intracellular concentration of putrescine, without affecting that of spermidine or spermine. On the other hand, CDDP (1.5  $\mu\text{M}$ , 72 hours) did not significantly change the polyamine level. CT-1 and CT-2 treatment schedules, but not CT-3, also caused a decline in the putrescine level (Figure 4). This suggests that longer treatment with 5-FU, but not with CDDP, may reduce the putrescine level.

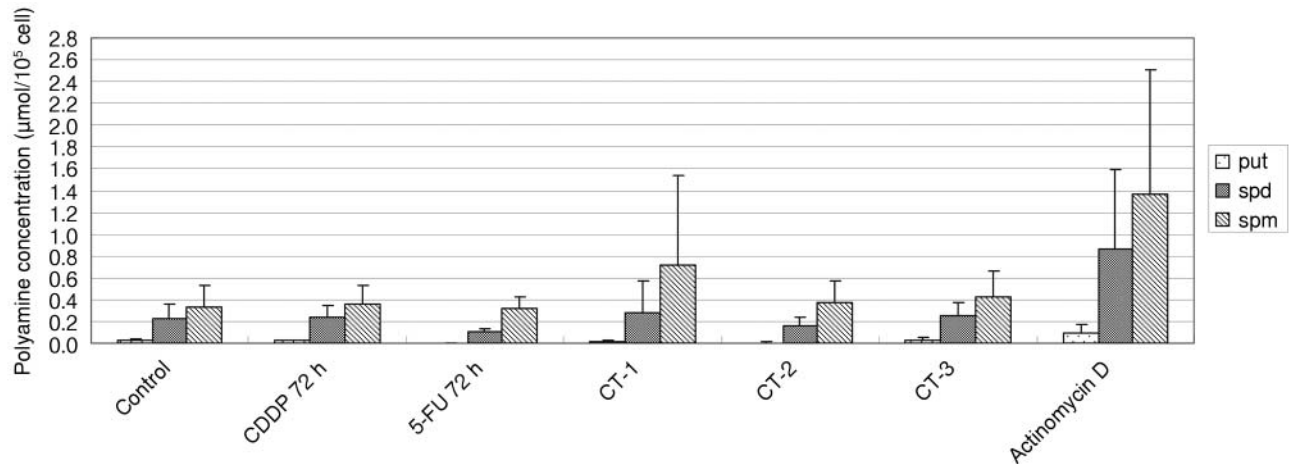


Figure 4. Effect on polyamine concentration of treatments of HSC-2 cells with CDDP and 5-FU. Near confluent HSC-2 cells were incubated for 72 hours alone (control), or with 1.5  $\mu$ M CDDP or 1.5  $\mu$ M 5-FU alone or applied in combination (CT-1) or sequentially (CT-2, CT-3), or actinomycin D (1  $\mu$ g/mL: 4 h) (positive control), and the intracellular polyamine concentration ( $\mu$ mol/10<sup>5</sup> cells) was determined by HPLC. Each value represents mean  $\pm$  S.D. from 3 independent experiments.

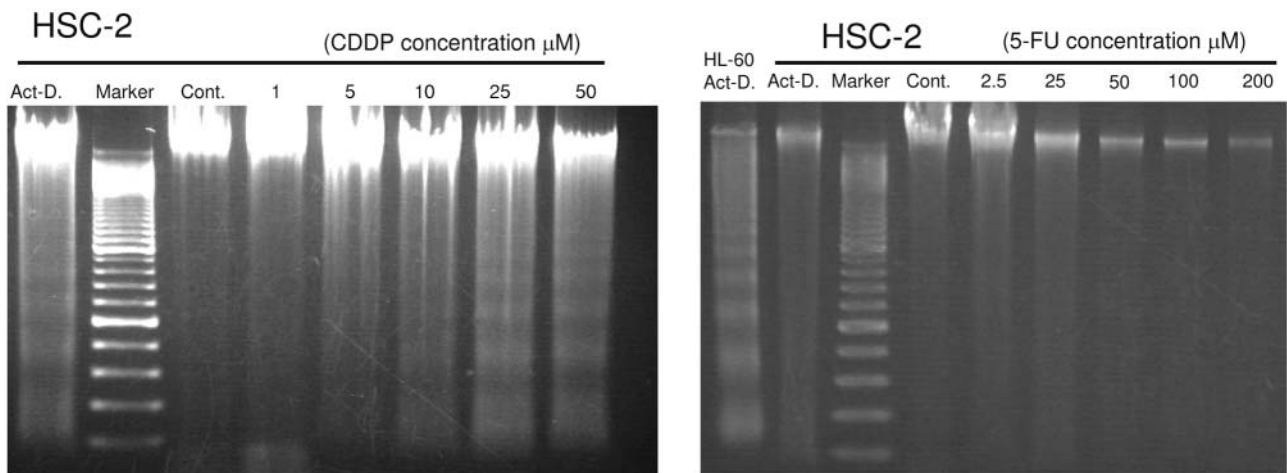


Figure 5. Induction of DNA fragmentation by extremely high concentrations of CDDP or 5-FU in HSC-2 cells. Near confluent HSC-2 cells were incubated for 48 hours alone (control) or with the indicated concentrations of CDDP or 5-FU, or with actinomycin D (100 ng/mL: 24 hours) and were assayed for DNA fragmentation. Act-D HL-60, DNA from apoptotic HL-60 cells induced by actinomycin D (1  $\mu$ g/mL: 6 hours).

## Discussion

To date, CDDP and 5-FU combination treatments have been reported to be one of the most active chemotherapeutic regimens for the patients with recurrent and disseminated head and neck squamous cell carcinoma (1-7). Repeated administration with low dose or continuous infusion of CDDP combined with 5-FU derivatives has been reported to induce the antitumor activity in Yoshida sarcoma-bearing rats (19). Our finding that simultaneous CDDP and 5-FU treatment for 72h (CT-1) showed higher cytotoxic activity against HSC-2 cells than CT-2 or CT-3 is in agreement with this. But, CDDP

treatment (24 hours) prior to 5-FU treatment (48 hours) has been reported to significantly enhance the induction of apoptosis in human oral cancer (B88) cells (12). Sequential treatment with 5-FU followed by CDDP, has also been reported to significantly reduce the tumor number and total tumor burden compared to these in control mice (20).

Many researchers have dealt with the optimization of the best sequence of combination treatment with CDDP and 5-FU *in vitro* and *in vivo* (8, 12, 13, 19-21). Treatment of MKN45 gastric cancer cells with low concentrations of CDDP and 5-FU has been reported to elevate the intracellular concentrations of Bax, cytochrome c, Fas and

caspase-3 proteins, but not that of caspase-8, suggesting the activation of the intrinsic apoptotic pathway (8). On the contrary, the present study shows that combination treatment with CDDP and 5-FU did not induce internucleosomal DNA fragmentation in HSC-2 cells. Furthermore, combination treatment of CDDP and 5-FU, which induced higher cytotoxicity than single treatments, activated caspase to lesser extent. These results suggest that combination treatment with CDDP and 5-FU may induce non-apoptotic cell death in HSC-2 cells. However, when HSC-2 cells were exposed for 48 hours with extremely high concentrations of CDDP (25, 50  $\mu$ M, about 7- to 15-fold higher than the  $CC_{50}$  value), internucleosomal DNA fragmentation was observed in HSC-2 cells (Figure 5).

Whether cells are committed to apoptosis or non-apoptotic cell death may depend on the concentrations of the test compounds used. The other factors that may distinguish these cell deaths are the type of both cells (22) and the test compounds used. Glioblastoma cells are easily committed to autophagy upon treatment with ceramide (anticancer agent), and radiation (23, 24). We have recently reported that codeinone (25), morphinone (26), and seven other simple cyclic  $\alpha,\beta$ -unsaturated ketones (2-cyclohexen-1-one, 2-cyclopenten-1-one, 4,4-dimethyl-2-cyclopenten-1-one, 2-cyclohepten-1-one,  $\alpha$ -methylene- $\gamma$ -butyrolactone, 5,6-dihydro-2*H*-pyran-2-one, methyl 2-oxo-2*H*-pyran-3-carboxylate) (27) activated caspase-3,8 and -9 very weakly. Trifluoromethylated  $\alpha$ -diketone ( $CF_3COCOPh$ ) and  $\alpha$ -hydroxy ketones ( $CF_3CH(OH)COPh$ ,  $CF_3CH(OH)COCH_2Ph$ ) showed higher tumor-specific cytotoxic activity than their corresponding non-fluorinated analogs, but failed to induce internucleosomal DNA fragmentation, or activate caspase-3. Electron microscopy of HL-60 cells treated with these ketones showed the development of autophagosomes in HL-60 cells, without the production of an apoptotic body, or affecting the mitochondria or cell surface microvilli (28). These data suggest that  $\alpha,\beta$ -unsaturated ketones may generally induce autophagic cell death.

The natural polyamines putrescine, spermidine and spermine are involved in cell proliferation, survival and death in multiple ways (14). We found that the intracellular concentration of putrescine declined by either 5-FU alone, CT-1 or CT-2 treatment schedule, in HSC-2 cells, although this treatment may induce non-apoptotic cell death. The correlation between the decline of putrescine and the induction of apoptosis was not confirmed. This may be due to the different cell system used: HL-60 (15, 16) and HSC-2 cells (present study). Further studies with various cell types are required. Polyamine replenishment interfered with 5-FU-induced apoptosis in colon carcinoma cell lines (29). CDDP was found to induce the expression of two enzymes critical to maintaining homeostasis, ornithine decarboxylase (ODC) and spermidine/spermine N1-acetyltransferase (SSAT) in kidneys of mice (30). This may be

a compensative or homeostatic reaction of cells in recovering from the CDDP-induced injury (31). We found that spermine was elevated when treating the cells with the CT-1 treatment schedule. This may be a cellular response to protect the cells from CDDP injury, since spermine is known to prevent endonuclease activation, and thymocytes incubated with guanlylhydrazine to deplete intracellular spermine exhibited spontaneous DNA fragmentation (32). It has been reported that the cellular proliferation and the expression of apoptosis marker proceeded simultaneously at the early stage of the induced oral oncogenesis in Syrian golden hamsters (33). This suggests that the balanced expression of cell proliferation maker (polyamine) and apoptosis maker (caspase activity) may be important for the chemotherapy of oral cancer.

## Conclusion

These results suggest that the combination of CDDP and 5-FU (CT-1) induces lower level of apoptotic cell death than actinomycin D (positive control) in HSC-2 cells, accompanied by selective decline in the intracellular concentration of putrescine. The causality of these factors remains to be investigated.

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