

Oxaliplatin in Treatment of the Cisplatin-resistant MKN45 Cell Line of Gastric Cancer

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Abstract. *Background and Aim:* The clinical efficiency of cisplatin (CDDP) against gastric cancer is often limited by the development of resistance. A third-generation platinum-containing agent, oxaliplatin (L-OHP), has been introduced for treating gastric cancer. Here, we studied oxaliplatin *in vitro* to reveal the mechanism of acquiring drug resistance and whether a cisplatin-resistant gastric cancer cell line has susceptibility to oxaliplatin. *Materials and Methods:* A cisplatin-resistant gastric cancer cell line (MKN45/CDDP/R1) was established by continuous exposure of MKN45 cells to cisplatin. The amount of excision repair cross-complementation group 1 (ERCC1) and glutathione-S-transferase (GST)- π mRNA was measured by real-time polymerase chain reaction (PCR). To examine the chemosensitivity to CDDP and L-OHP in MKN45 and MKN45/CDDP/R1 cells, a collagen gel droplet-embedded culture drug sensitivity test (CD-DST) was performed. The intracellular concentration of CDDP and L-OHP were also measured to see if the drugs would be taken up by these cell lines. *Results:* The MKN45/CDDP/R1 cell line was resistant to CDDP. The ERCC1 and GST- π mRNA was significantly increased in MKN45/CDDP/R1 cells, indicating that the cells acquired resistance to CDDP. Intracellular CDDP was not detected in MKN45/CDDP/R1 cells up to 48 h after incubation, indicating that uptake and efflux processes of CDDP were altered in these cells. MKN45/CDDP/R1 cells were still susceptible to L-OHP. The intracellular concentration of CDDP but not L-OHP was significantly

reduced in MKN45/CDDP/R1 cells. *Conclusion:* We established a CDDP-resistant cell line using MKN45 cells, in which ERCC1 and GST- π were increased. This cell line showed susceptibility to the new generation platinum agent L-OHP, suggesting this anticancer agent could be used in second-line treatment of patients with CDDP-resistant gastric neoplasms.

Cisplatin (CDDP) is the most frequently used chemotherapeutic agent for various types of advanced cancer and is used in combination regimens. Gastric cancer is one of the major causes of cancer-related mortality worldwide (1). In a phase II study of CDDP in advanced gastric cancer, the treatment response rate was 22% (2) and cases of complete remission are rare. Several combination chemotherapy regimens such as FAM (5-fluorouracil, adriamycin and mitomycin C) have been used to improve the treatment outcomes (3). In a non-randomized phase II study on advanced gastric cancer, the FAM regimen achieved an objective partial response rate of 42% (4). Some CDDP-based combination chemotherapy regimens have also shown high response rates (5, 6). However, inherent or acquired resistance to CDDP reduces its efficacy (7, 8). The mechanism of CDDP resistance *in vivo* has been studied and laboratory studies on tumor tissues and cell lines suggest that resistance to CDDP is nearly always multifactorial (9). These factors include impaired cellular uptake of CDDP (10), enhanced intracellular detoxification by glutathione and metallothionein systems (11), altered patterns of DNA platination, increased tolerance to platinum-DNA damage and enhanced repair of DNA damage (12).

Oxaliplatin (L-OHP) is a third-generation CDDP analog with a 1,2-diaminocyclohexane (DACH) carrier ligand (13). Its main mode of action is mediated by the formation of DACH-platinum adducts. The mechanism is still not fully clear but it seems that L-OHP injures the DNA of tumor cells, thus triggering the induction of apoptosis through the pathway involving cytochrome *c*, which is

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found in mitochondria, and where the drug acts mainly to induce DNA fragmentation and chromatin condensation (14, 15).

In this study, we developed a CDDP-resistant cell line from a human gastric cancer cell line by repeated exposure of cells to stepwise-increasing concentrations of CDDP. The mechanism of acquisition of drug resistance and whether this CDDP-resistant gastric cancer cell line had susceptibility to L-OHP was investigated.

Materials and Methods

Cell line and drugs. MKN45, a human stomach adenocarcinoma cell line, was purchased from the Japanese Riken Cell Bank (Tsukuba, Japan). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a balanced air humidified incubator with an atmosphere of 5% CO₂ (16). CDDP was purchased from Bristol-Myers Co. (Tokyo, Japan). L-OHP was a gift from Yakult (Tokyo, Japan).

Establishment of a CDDP-resistant subline from the MKN45 cell line. A CDDP-resistant subline was developed by continuous exposure to CDDP starting at 0.1 µg/ml and increasing in a stepwise manner to 1 µg/ml. The resistant subline, which was maintained in the medium containing CDDP for more than 10 months, was considered established. The CDDP-resistant MKN45 subline is named MKN45/CDDP/R1 throughout the text. Experiments with this subline were performed after maintenance in CDDP-free medium for 2-3 weeks.

Measurement of the resistance against CDDP and L-OHP. Cell viability was evaluated using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the cells were treated for 48 h with graded concentrations of CDDP (0-50 µg/ml) or L-OHP (0-5 µg/ml). After the treatment, CellTiter-Glo reagent was added and the cells incubated for 10 minutes at room temperature. The luminescence was measured by a luminometer. The values from the treated cells were compared with the values generated from the untreated cells and reported as percentage viability. Each experiment was performed in triplicate.

The 50% inhibitory concentration (IC₅₀) for each particular agent was defined as the drug concentration which resulted in a 50% reduction in luminescence compared to the untreated control. IC₅₀ values were determined directly from semilogarithmic dose-response curves.

Real-time PCR. The level of mRNA expression of *glutathione-S-transferase (GST)-π* and *excision repair cross-complementation group 1 (ERCC1)* were analyzed using real-time quantitative reverse-transcription PCR (real-time PCR). Total RNA was prepared from MKN45 and MKN45/CDDP/R1 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared using the cDNA Synthesis Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with the random hexamers using 1 µg of total RNA (17, 18). Real-time PCR was carried out using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). A TaqMan probe and primer set designed to amplify the *GST-π* and

ERCC1 gene constructs was used to quantify 5 µl of cell lysate in a total PCR volume of 50 µl. Reagents utilized included the TaqMan 2× Universal Master Mix containing 1× TaqMan buffer A, 5 mM MgCl₂, 400 µM dUTP, 200 µM dATP, dCTP, dGTP (each), 8% glycerol, AmpliTaq Gold (0.025 U/µl), AmpErase UNG (0.01 U/µl), 300 nM of each forward and reverse primer, and 100 nM probe. The kit was utilized according to manufacturer's directions. The PCR cycling conditions were performed for all samples as follows: 50°C, 2 min for AmpErase UNG incubation, 95°C, 10 min for AmpliTaq Gold activation, and 50 cycles for the melting (95°C, 15 s) and annealing/extension (60°C for 1 min) steps. PCR reactions for each template were carried out in duplicate in 96-well plates. Significant contamination with genomic DNA was excluded by amplifying non reverse-transcribed RNA. Data analyses were performed with the ABI PRISM sequence detection software.

Measurement of the intracellular concentration of CDDP and L-OHP. The parental cell line and the CDDP-resistant cell clone were treated with CDDP (1 µg/ml) or L-OHP (0.1 µg/ml). The cells were harvested following 12, 24, 48, 72, or 96 h CDDP exposure, or following 24, 48, or 72 h L-OHP exposure. Cells were centrifuged into a pellet and lysed in 0.5 ml 0.1% Triton[®] X-100 and 0.2% nitric acid overnight and sonicated for 1 min. Platinum in the cell lysate was measured by graphite furnace atomic absorption spectrophotometry with Zeeman background correction (Perkin-Elmer SIMAA6000, Perkin-Elmer Japan Co., Ltd., Osaka, Japan) (19).

Statistics. Results are expressed as means±SD. Multiple groups were compared by Dunnett's test after one-way ANOVA. Single comparisons were performed by a non-parametric test (unpaired Student's *t*-test). Differences were considered significant for *p*<0.01.

Results

Establishment of CDDP-resistant MKN cells (MKN45/CDDP/R1). A CDDP-resistant cell line from MKN45 cells (MKN45/CDDP/R1) was established by continuous exposure to CDDP starting at 0.1 µg/ml and increasing in a stepwise manner to 1 µg/ml. The established MKN45/CDDP/R1 cell line was resistant up to 50 µg/ml of CDDP (Figure 1). On the other hand, MKN45 cells were sensitive to CDDP.

ERCC1 and GST-π mRNA in MKN45 and MKN45/CDDP/R1 cells. The expression profiles of the resistance-related genes were examined in the parental MKN45 and the established MKN45/CDDP/R1 cells by real-time PCR. The resistance-related genes examined were *ERCC1* and *GST-π*. The amount of *ERCC1* mRNA was significantly higher in MKN45/CDDP/R1 cells compared to the parental MKN45 cells (Figure 2). *GST-π* mRNA was also greater in MKN45/CDDP/R1 cells (data not shown). These data indicate that *ERCC1* and *GST-π* might be involved in CDDP resistance in the MKN45/CDDP/R1 cell line.

Intracellular concentration of CDDP. When the cells were incubated with CDDP (1.0 µg/ml), the intracellular

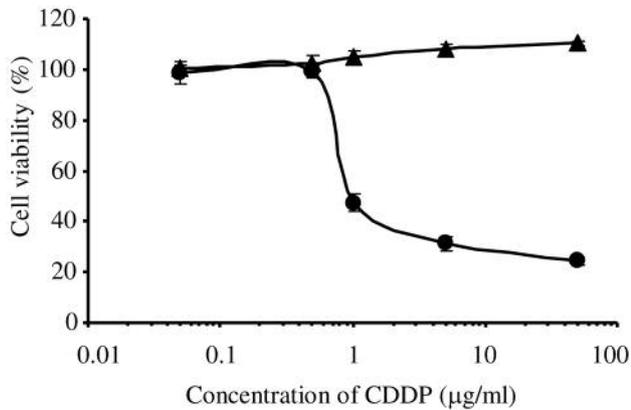


Figure 1. Measurement of the resistance against cisplatin (CDDP). A CDDP-resistant cell line from MKN45 cells (MKN45/CDDP/R1) was established by continuous exposure to CDDP. The established MKN45/CDDP/R1 cell line (▲) was resistant up to 50 µg/ml of CDDP. On the other hand, the IC_{50} of CDDP against MKN45 cells was 0.98 µg/ml (●).

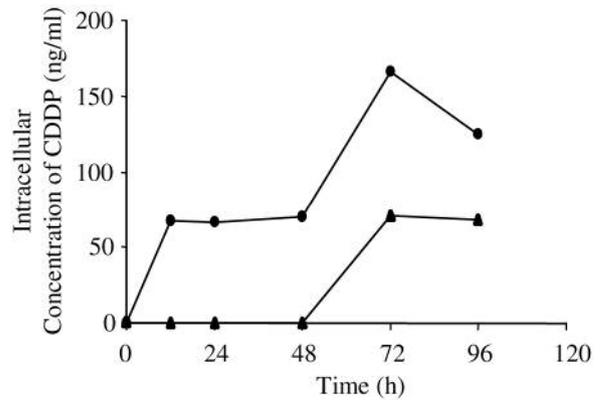


Figure 3. Intracellular concentration of CDDP. The intracellular concentration of CDDP in MKN45 cells (●) was 70 ng/ml at 12 h and increased up to 166 ng/ml at 72 h on incubation with CDDP (1.0 µg/ml). On the other hand, the intracellular concentration of CDDP in MKN45/CDDP/R1 cells (▲) was not detectable up to 48 h but increased to 71 ng/ml at 72 h.

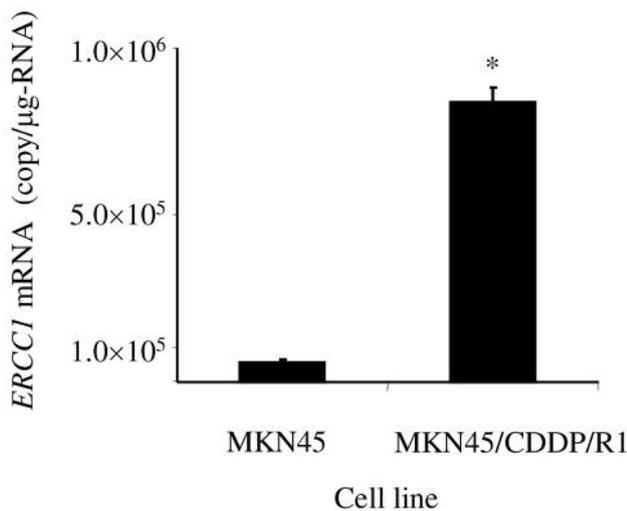


Figure 2. ERCC1 mRNA in MKN45 and MKN45/CDDP/R1 cells. The amount of ERCC1 mRNA significantly increased in MKN45/CDDP/R1 cells compared to MKN45 cells (** $p < 0.01$ vs. MKN45).

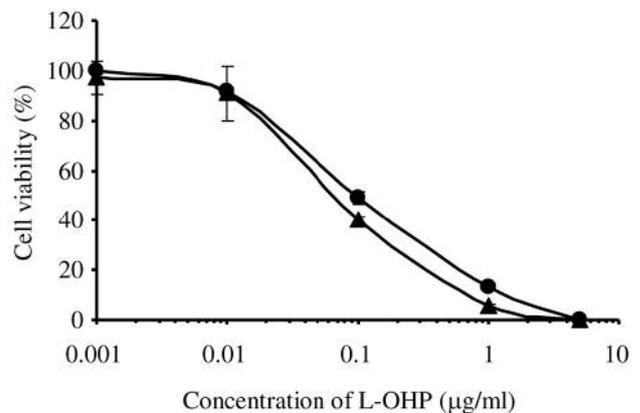


Figure 4. The effect of oxaliplatin (L-OHP) on MKN45 and MKN45/CDDP/R1 cells. MKN45 (●) and MKN45/CDDP/R1 (▲) cells were cultured with L-OHP (0.001 - 5 µg/ml). The IC_{50} of L-OHP in MKN45 and MKN45/CDDP/R1 cells was 66 and 96 µg/ml, respectively.

concentration of CDDP in MKN45 was 70 ng/ml at 12 h and increased up to 166 ng/ml at 72 h (Figure 3). On the other hand, the intracellular concentration of CDDP was 0 ng/ml in MKN45/CDDP/R1 cells up to 48 h after the incubation, suggesting reduced accumulation (reduced influx or increased efflux) of CDDP in MKN45/CDDP/R1 cells.

The effect of L-OHP on MKN45 and MKN45/CDDP/R1 cells. Regarding the effect of L-OHP, the IC_{50} of L-OHP in

MKN45 and MKN45/CDDP/R1 cells was 66 and 96 µg/ml respectively (Figure 4). When the cells were incubated with L-OHP (0.1 µg/ml), the intracellular concentration of L-OHP in MKN45 and MKN45/CDDP/R1 cells was 165.3 and 136.3 ng/ml respectively (Figure 5). The concentration of L-OHP in the cells was not different. These data indicate that L-OHP is able to penetrate the cells and is not expelled from the cells even in the high ERCC and GST- π expressing MKN45/CDDP/R1 cell line.

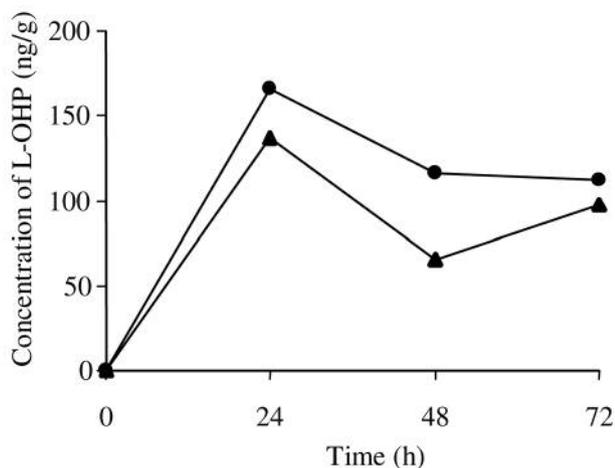


Figure 5. Intracellular concentration of L-OHP. When the cells were incubated with L-OHP (0.1 μ g/ml), the intracellular concentration of L-OHP in MKN45 (●) and MKN45/CDDP/R1 cells (▲) was 165.3 and 136.3 ng/ml respectively at 24 h. The concentration of L-OHP in the cells was not significantly different up to 72 h.

Discussion

Gastric cancer is the second leading cause of cancer death worldwide and continues to carry a poor prognosis, making it a therapeutic challenge for oncologists. 5-Fluorouracil (5-FU) and CDDP are considered mainstays in the treatment of gastric cancer. L-OHP is a third-generation platinum drug and it is used in metastatic colorectal cancer with 5-FU and leucovorin (LV) (20). L-OHP was evaluated in combination with various 5-FU-LV regimens for gastric cancer. The 5-FU-LV-L-OHP combination yielded response rates in the range of 38-54% and median overall survival times of approximately 10 months (21).

Nucleotide excision repair (NER) is important in the repair of bulky covalent lesions within DNA. The role of DNA repair in the development of cellular and clinical resistance to anti cancer chemotherapy is an evolving science (22). The human excision repair gene, *excision repair cross-complementation group 1* (*ERCC1*), is one of the critical repair genes in NER. Overexpressions of *ERCC1* and other NER genes are associated with the removal of lesions on DNA produced by chemotherapeutic drugs and clinical resistance to CDDP (12). Removal of CDDP-induced DNA lesions is believed to occur mainly by NER (23). High tumor tissue levels of *ERCC1* mRNA have been correlated with poor survival and response of gastric cancer patients treated with CDDP and 5-FU (24), whereas low mRNA levels were associated with clinical sensitivity (22). In this study, *ERCC1* was significantly increased in CDDP-resistant cells (Figures 1 and 2). Xu *et al.* (25) reported that an established CDDP-resistant gastric cell

line was cross resistant to carboplatin, heptaplatin, doxorubicin, mitomycin C and 5-FU compared to the parental cell line. However, in the present study, L-OHP was still active in high *ERCC1*-expressing CDDP-resistant gastric cells at physiological concentrations (Figure 4). The clinically relevant plasma concentration of L-OHP is 2 μ M in patients under treatment with L-OHP.

The glutathione-S-transferases (GSTs) are a complex supergene family of enzymes (26). These enzymes are multifunctional enzymes that play an important role in cellular detoxification (27). It has been reported that the expression of *GST- π* is significantly related to the sensitivity of gastric cancer to CDDP (27). *GST- π* in tumor cells has been suggested to relate to CDDP resistance as determined in studies using culture cells derived from various organs *in vitro* (28). In this study, *GST- π* expression was increased in CDDP-resistant cells (Figure 2). However, L-OHP was effective against CDDP-resistant cells.

Elevated *ERCC1* and *GST- π* levels appear to be implicated in the mechanism of CDDP resistance in CDDP-resistant MKN45 cells (Figure 2). Hector *et al.* reported that *ERCC1* mRNA was elevated in L-OHP- and CDDP-resistant cells and that L-OHP resistance might be mediated by similar mechanisms of reduced drug accumulation and DNA-Pt adduct formation as in resistance to CDDP (29). On the other hand, the *ERCC1* mRNA level was not increased in an L-OHP-resistant ovarian carcinoma cell line (30). Although resistance to CDDP in a clone was associated with significantly reduced CDDP uptake (Figure 3), when L-OHP was applied to the CDDP-resistant MKN45 cells, the intracellular concentration of L-OHP was the same as that in the parental MKN45 cells (Figure 5). Arnould *et al.* (31) have reported that *ERCC1* expression was predictive of L-OHP sensitivity and that GST activity was not correlated to L-OHP cytotoxicity in colonic cancer cell lines. These data indicate that the mechanisms of uptake and efflux processes of CDDP and L-OHP are different in CDDP-resistant cells and that *ERCC1* and/or *GST- π* are not always involved in L-OHP resistance. L-OHP may be useful for clinical application against gastric cancer due to its different antitumor action as compared to CDDP.

An obvious limitation of this study is that the kinetics mechanisms may be dependent on the cell lines tested. In this study, we used MKN45 cells as a representative poorly differentiated gastric adenocarcinoma. National Cancer Institute (NCI) anticancer drug screening panels comparing L-OHP and other platinum agents have shown that CDDP and L-OHP have different sensitivity profiles in mouth squamous cell and ovarian cancer cell lines, suggesting that the two complexes may have different mechanism(s) of action and/or resistance (32). Recently, Fink *et al.* (33) have shown that colon carcinoma cell lines either defective in hMLH1 or hMSH2 MMR enzymes are 1.5- to 2-fold more resistant to

CDDP, but display little or no resistance to L-OHP. Further studies are needed in gastric cancer cells, including establishing other CDDP-resistant cell lines and L-OHP-resistant cells, to clarify the unidentified mechanisms of CDDP and L-OHP resistance in gastric cancer in detail.

In summary, we established a CDDP-resistant cell line (MKN45/CDDP/R1), which is useful as an *in vitro* model for studying the resistance mechanism of CDDP and for studying the effects of other anticancer drugs for gastric cancer under CDDP resistance. L-OHP is effective at physiological concentrations, at least on MKN45/CDDP/R1 cells.

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