

Cyclooxygenase-2 Gene Induction Causes CDDP Resistance in Colon Cancer Cell Line, HCT-15

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Abstract. Drug resistance to cisplatin (CDDP) would represent a major obstacle for cancer therapy. The adenosine triphosphate (ATP) binding cassette (ABC) family of transport proteins, such as the 170 kDa P-glycoprotein (multidrug resistance gene-1; MDR-1) and the 190 kDa multidrug resistance-associated proteins (MRPs), are associated with multidrug resistance, including resistance to CDDP. The purpose of the present study was to investigate the relationship between cyclooxygenase-2 (COX-2) expression and the level of chemosensitivity to CDDP. We established the COX-2-overexpressed colon cancer cell line TR-5 from HCT-15 cells. We quantified the expression of m-RNA for MRP-1 and MDR-1 by a real-time PCR method, determining that the values of each gene/standardized GAPDH in HCT-15 and TR-5 were 2.3 ± 0.4 and 6.1 ± 0.5 in MRP-1 ($p < 0.02$) and 9.0 ± 4.8 and 3.6 ± 0.5 in MDR-1, respectively. With respect to chemosensitivity, survival rates for 3 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ of CDDP were $81.5 \pm 12.2\%$ and $26.1 \pm 11.7\%$ ($\text{IC}_{50} = 6.5 \mu\text{g/ml}$) for HCT-15 and $96.6 \pm 1.7\%$ and $77.4 \pm 4.9\%$ ($\text{IC}_{50} = 18.5 \mu\text{g/ml}$) for TR-5, respectively, thus TR-5 showed higher resistance to CDDP than HCT-15 did with statistical differences. We also demonstrated a successful re-sensitization to CDDP toxicity in TR-5 by means of the COX-2 selective inhibitor JTE-522, 4-(4-cyclohexyl-2-methyl-1, 3-oxazol-5-yl)-2-fluorobenzene sulfonamide, which markedly decreased the IC_{50} of CDDP for TR-5 (from $17.3 \pm 2.6 \mu\text{g/ml}$ to $8.6 \pm 2.5 \mu\text{g/ml}$). In conclusion, COX-2 overexpression induced increased MRP-1 expression in a colon cancer cell line, TR-5, resulting in chemoresistance to CDDP that was approximately triple the level of chemoresistance observed in the original HCT-15 cells line, as measured by calculation of the IC_{50} . We also confirmed the efficacy of pretreatment of TR-5 cells with the

COX-2 selective inhibitor JTE-522 in restoring chemosensitivity of these cells to CDDP, suggesting a strategy for overcoming drug resistance to CDDP.

Since cis-diamminedichloroplatinum II (cisplatin; CDDP) is one of the most effective drugs against colon cancer, the development of drug resistance to CDDP would represent a major obstacle to the effective treatment of this cancer. Multidrug resistance has been shown to be conferred by different integral membrane proteins, including the 170 kDa P-glycoprotein (multidrug resistance gene-1; MDR-1) and the 190 kDa multidrug resistance-associated proteins (MRPs), which belong to the adenosine triphosphate (ATP) binding cassette (ABC) family of transport proteins (1-3). MDR-1 contributes to multidrug resistance through its action as an efflux pump for chemotherapeutic drugs (1). The recent discovery that overexpression of cyclooxygenase-2 (COX-2) increases intracellular production of MDR-1 *in vitro* has given rise to speculation that selective COX-2 inhibitors could enhance the antitumor activity of chemotherapeutic agents by modifying MDR-1 expression (4, 5).

Cyclooxygenase (COX) converts arachidonic acid to prostaglandin (PG) H₂, which serves as the precursor for other PGs and thromboxane (TX) (6). COX has two major isoforms: COX-1 and COX-2. Recently, COX-2 has been found to be up-regulated under inflammatory conditions *in vitro* and *in vivo*. Enhanced expression of COX-2, but not COX-1, has been found in various cancers (7). Through their activity as COX inhibitors, non-steroidal anti-inflammatory drugs are reported to have the potential to reduce the risk of colon cancer by 40-50% (8, 9), suggesting that COX-2-mediated PG biosynthesis may be involved in the development of cancer. The role of COX-2 in both carcinogenesis and cancer biology is now under investigation.

The purpose of the present study was to investigate the relationship between COX-2 expression and the level of chemosensitivity to CDDP. We established the COX-2-overexpressed colon cancer cell line TR-5 from HCT-15 cells, which do not express COX-2 m-RNA and protein (Figure 1).

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Key Words: Colon cancer cell, cisplatin, cyclooxygenase-2, MDR-1, MRP-1.

We evaluated chemosensitivity to CDDP in both TR-5 and HCT-15 cells, and we also quantified the expressions of messenger RNA for MDR-1 and MRP-1, which are reported to be molecules related to CDDP resistance (10-15). In addition, we assessed the efficacy of the COX-2 selective inhibitor JTE-522 in overcoming drug resistance to CDDP resulting from COX-2 gene induction in TR-5 cells.

Materials and Methods

Drugs and cell lines. Cisplatin (CDDP) was purchased from Nihon Kayaku (Tokyo, Japan). JTE-522, 4-(4-cyclohexyl-2-methyl-1, 3-oxazol-5-yl)-2-fluorobenzenesulfonamide, which is reported to possess selective inhibitory activity on human COX-2 with potent anti-inflammatory activities and very little gastrointestinal ulcerogenicity, suppressing PGE2 production from arachidonic acid by inhibition of COX-2, was kindly provided by Japan Tobacco, Inc. (Tokyo, Japan) (16). JTE-522 was dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan), which was diluted to a final concentration of less than 0.1% DMSO so that DMSO would not affect cell toxicity. Two colon cancer cell lines, HCT-15 and HT29, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and maintained in RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) with 1% antibiotics and 10% fetal bovine serum (FBS; F4010 Sigma, St. Louis, MO, USA). All incubations were carried out at 37°C in a humidified atmosphere of 95% air and 5% CO₂. HT29 expresses COX-2 mRNA moderately, while HCT-15 does not express COX-2 m-RNA (Figure 1).

COX-2 transfection into colon cancer cells, HCT-15 (Figure 2). Complementary DNA for human COX-2 was obtained by PCR cloning. The reaction was performed using Pfx DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA) with primers (Sigma Genosys Japan Co. Ltd, Hokkaido, Japan); 5'-CTGAATTCAGACGCCCTCAGACAGCAAAGC-3' (*Eco*RI linker was underlined) and 5'-CTAAGCTTGATCATTAGACTTCTA CAGTTC-3' (*Hind*III linker was underlined), and using cDNA from HT-29 cancer cells as a template. The PCR product was purified and subcloned into the *Eco*RI-*Hind*III site of an eukaryotic expression vector, pcDNA3.1 (Invitrogen Corp.), and the entire sequences were verified by sequencing again. COX-2 cDNA subcloned in pcDNA3.1 expression vector (described above) was stably transfected into HCT-15 cells by the electroporation method (Figure 2). These cells were selected in the presence of Geneticin (G418) (Invitrogen Corp.), at a concentration of 0.8 mg/ml in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated FBS. After three weeks of culture, 10 colonies were identified and expanded monoclally. Several clones demonstrated overexpression of COX-2 m-RNA; TR-5, which had an enhanced level of COX-2 m-RNA, was chosen for the present study (Figure 3).

Drug treatment schedule. 3 X 10³ HCT-15 or TR-5 cells/well were incubated in 96-well microplates, cultured with control medium plus FCS for 24 h, and exposed to various concentrations of CDDP for 48 h before the end point. IC₅₀ was calculated from the concentration-survival curve. In the experiments involving CDDP/JTE522 combination treatment, 3 X 10³ HCT-15 and TR-5 cells/well were incubated in 96-well microplates, cultured with control medium plus FCS for 24 h, and exposed to 100 µM of JTE522 for 24 h followed by

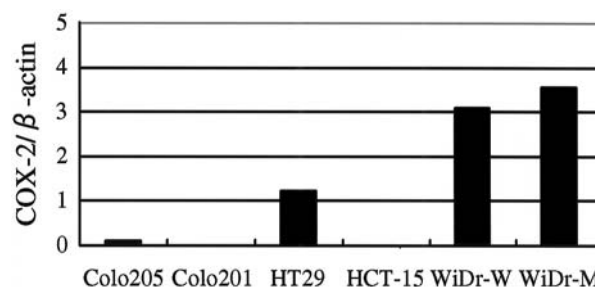


Figure 1. As demonstrated by their COX-2/β-actin ratios, human colon cancer cell line HCT-15 did not express cyclooxygenase-2 (COX-2) messenger RNA, while HT29 cells showed moderate expression of COX-2 messenger RNA. Messenger RNA was quantified using a real-time PCR method.

CDDP treatment as described above. The IC₅₀ of CDDP/JTE522 was calculated from the concentration-survival curve.

MTT assay. The viability and the survival of cancer cells *in vitro* were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay developed by Mosmann *et al.* with modifications (17). This method evaluates the number of viable cells by measuring the activity of mitochondrial succinate dehydrogenase. At the end of the incubation period, 0.4% MTT and 0.1 M sodium succinate, each dissolved in 10 ml phosphate-buffered saline and filtered through a 0.45-mm membrane filter (Millipore, Bedford, MA, USA), were added to each well, and the plates were incubated for an additional 3 h at 37°C. DMSO was then added to a final volume of 150 µl/well and the plates were mechanically shaken for 10 min on a mixer (Model 250, Sonifier, Branson, MO, USA) to dissolve the MTT-formazan product. Absorbance (Abs) at 540-630 nm was determined on a model EAR 340 easy reader (SLT-Labinstruments, Salzburg, Austria). Every experiment was repeated at least three times to calculate the mean and standard deviation values of the survival ratios, which were calculated using the following formula:

Survival ratio = (Abs of treated cells - Abs of the blank) / (Abs of control cells - Abs of the blank).

RNA extraction and cDNA synthesis. Total RNA was isolated using an RNeasy mini kit (QIAGEN Inc., Chatsworth, CA, USA), and DNase treatment was performed using the RNase-Free DNase Set (QIAGEN Inc.), following the manufacturer's instructions.

Reverse transcription of up to 10 µg total RNA was carried out in a total volume of 100 µl containing 250 pmol oligo (dT) 18, 80 U rRNasin ribonuclease inhibitor (Promega, Madison, WI, USA), and 500 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and 0.5 mM dNTPs. Total RNA and oligo (dT) 18 solutions were initially heated at 70°C for 10 min and immediately chilled on ice. The other reagents were then added and incubated for 15 min at 30°C and then for 60 min at 42°C.

Primers and TaqMan probes. Primers and TaqMan probes for GAPDH, MDR-1 and MRP-1 were designed using Primer Express software (PE Biosystems, Foster City, CA, USA). Primers and TaqMan probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from PE Biosystems. Probes were labeled with a reporter dye (FAM) at the 5' end of the oligonucleotide and a quencher dye (TAMRA) at the 3' end. Sequences of primers and probes used were:

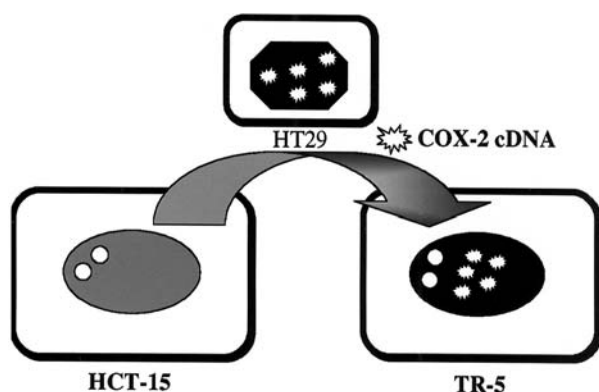


Figure 2. Transfection by COX-2 cDNA. Complementary DNA for human COX-2 was obtained by PCR cloning using HT-29 colon cancer cell cDNA as a template. The PCR product was purified and the subcloned COX-2 cDNA in pcDNA3.1 expression vector was stably transfected into HCT-15 cells by the electroporation method.

GAPDH-forward primer, GAAGGTGAAGGTCGGAGTC; GAPDH-reverse primer, GAAGATGGTGATGGGATTTC; and GAPDH-probe, CAAGCTTCCCGTCTCAGCC (from GenBank accession no. M33197). MRP-1-forward primer, CCTGCAGCAGAGAGGTCTTTTC; MRP-1-reverse primer, GGCATATAGGCCCTGCAGTTC; and MRP-1-probe, CAAAGACGCCGCTTGGTGTG (from GenBank accession L05628). MDR-1-forward primer, GTGGTGTTCAGAAATGGCAGAGT; MRP-1-reverse primer, AGCCTGGACACTGACCATTGA; and MRP-1-probe, AGCATGGCAGCATCAGCAGCT (from GenBank accession NM000927).

PCR procedure. Quantification of target cDNA (MDR-1 and MRP-1) and internal reference gene (GAPDH) was conducted using a fluorescence-based real-time PCR method (TaqMan PCR on an ABI PRISM 7700 Detection System, PE Biosystems) (18). PCR was carried out in a 25 μ l reaction volume containing cDNA equivalent to 1-10 ng total RNA, 200 nM of each primer, 100 nM probe and 12.5 μ l TaqMan universal PCR Master Mix (containing 1x TaqMan buffer, 200 μ M dATP, dCTP, dGTP and 400 μ M dUTP, 5 mM $MgCl_2$, 1.25 U AmpliTaqGold, 0.5 U AmpErase UNG) (PE Biosystems). Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Relative quantification of gene expression was performed using the relative standard curve method. The standard curve was created automatically by ABI PRISM 7700 Detection System software by plotting the threshold cycle (CT) against each input amount of control total RNA (16, 4, 1, 0.25, 0.063, 0.016 and 0.0039 ng total starting RNA) prepared from A549 human lung cancer cells (American Type Culture Collection, Manassas, VA, USA). The coefficient of linear regression (r) for each standard curve was more than 0.990. For each unknown sample, the relative amount was calculated using linear regression analysis from the respective standard curve. A relative target gene expression value was obtained by division of the target gene value by the GAPDH value as an internal reference gene.

Data analysis. Statistical analysis was performed using Fisher's direct probability, Chi-square test, or Student's *t*-test. Significance levels of $p < 0.05$ or < 0.02 were regarded as statistically significant.

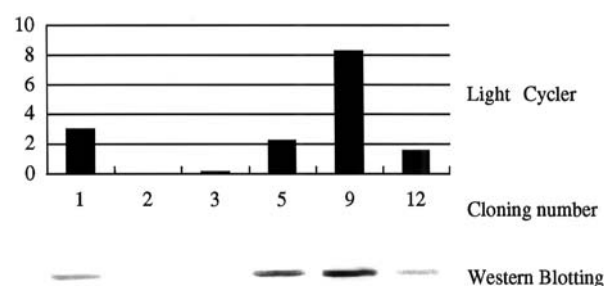


Figure 3. Expression of COX-2 m-RNA and protein in transfected HCT-15. After three weeks of culture following cDNA transfection, 10 colonies were identified and expanded monoclally. Several clones demonstrated overexpression of COX-2 m-RNA. The number 5 clone (TR-5), which had an enhanced level of COX-2 m-RNA, was chosen for the present study.

Results

Establishment of COX-2-overexpressed cells from HCT-15. TR-5 was established from HCT-15 through transfection by COX-2 genes derived from HT29 colon cancer cells; COX-2 m-RNA was not detected in the original HCT-15 cells (Figure 1). Ten colonies were identified and expanded monoclally three weeks after transfection by the vector described in "Materials and Methods" using the electroporation method (Figures 2, 3). Several clones were confirmed to be overexpressing COX-2 m-RNA and protein. The number 5 clone (TR-5) was chosen for the present study (Figure 3).

Altered m-RNA expression of MRP-1 and MDR-1 after transfection by the COX-2 gene. The expression of MRP-1 and MDR-1 m-RNA was quantified by a real-time PCR method, as described in "Materials and Methods". Quantified ratios (each gene/standardized GAPDH) for HCT-15 and TR-5 were 2.33 ± 0.4 and 6.07 ± 0.5 for MRP-1 and 8.97 ± 4.8 and 3.6 ± 0.5 for MDR-1, respectively (Figure 4). Increased MRP-1 expression by TR-5 demonstrated statistical significance compared with MRP-1 expression by HCT-15 ($p < 0.02$), indicating that one cause for the decreased sensitivity to CDDP in TR-5 might be MRP-1 overexpression through transfection by the COX-2 gene into TR-5.

Decreased sensitivity to CDDP in TR-5 compared to HCT-15 in vitro. CDDP antitumor activity was evaluated by the MTT assay, which revealed that CDDP showed dose-dependent antitumor activity in TR-5 and HCT-15 cells (Figure 5). Treatment with 3 μ g/ml, 10 mg/ml and 30 mg/ml of CDDP elicited $81.5 \pm 12.2\%$, $26.1 \pm 11.7\%$ and $11.3 \pm 0.4\%$ survival rates in HCT-15 cells and $96.6 \pm 1.7\%$, $77.4 \pm 4.9\%$ and $16.0 \pm 10.0\%$ survival rates in TR-5 cells, respectively. A statistically significant difference was observed between HCT-15 and TR-5 survival rates for cells treated with 3 and 10 mg/ml of CDDP ($p < 0.05$). We also calculated the IC_{50} s of CDDP for each cell line. The IC_{50} was 6.5 mg/ml for HCT-15 and 18.5 mg/ml for TR-5; this difference was statistically significant ($p < 0.05$) (Figure 6).

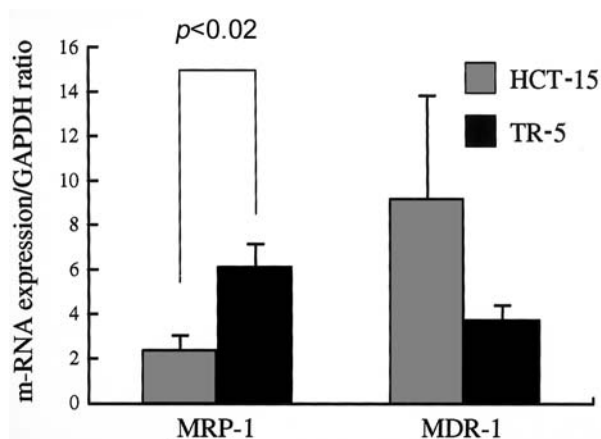


Figure 4. Quantification of m-RNA for chemoresistance-related genes, MDR-1 and MRP-1. Target cDNA (MDR-1 and MRP-1) and internal reference gene (GAPDH) were quantified using a fluorescence-based real-time PCR method (TaqMan PCR on an ABI PRISM 7700 Detection System). Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. The standard curve was created as described in "Materials and Methods". A relative target gene expression value was obtained by division of the target gene value by the GAPDH value as an internal reference gene.

Successful re-sensitization to CDDP toxicity in TR-5 by means of the COX-2 inhibitor, JTE522. The IC₅₀s of CDDP treated with/without 100µg/ml of JTE522 were calculated for HCT-15 and TR-5. CDDP showed a remarkable efficacy following TR-5 pre-treatment with JTE522, as demonstrated by a decline in IC₅₀ from 17.3±2.6 mg/ml to 8.6±2.5 mg/ml. Conversely, HCT-15 pre-treatment by JTE522 caused only a slight decrease in the IC₅₀ value of CDDP from 6.7±0.3 mg/ml to 4.9 ±1.0 mg/ml. These data demonstrate that, in the absence of JTE-522 pre-treatment, the IC₅₀ value for TR-5 was approximately triple the IC₅₀ value for HCT-15; pre-treatment with JTE-522 successfully re-sensitized the TR-5 response to CDDP to almost the same level of sensitivity as the HCT-15 response to CDDP (Figure 6).

Discussion

CDDP is a widely used chemotherapeutic agent that is effective as a single drug or in combination with other chemotherapeutic agents against colon cancer and other malignant solid tumors, including cancers of the head and neck, lung, ovaries and esophagus (19-21). The development of drug resistance to CDDP would represent a major obstacle to clinically effective treatment against cancer. Several studies using *in vitro* cell lines suggest that acquired cisplatin resistance is associated with defects in the apoptotic program, decreased cisplatin accumulation and increased drug inactivation by several mechanisms (22-24). Other studies have reported that altered molecules expressions such as c-myc, the adenosine triphosphate (ATP) binding cassette

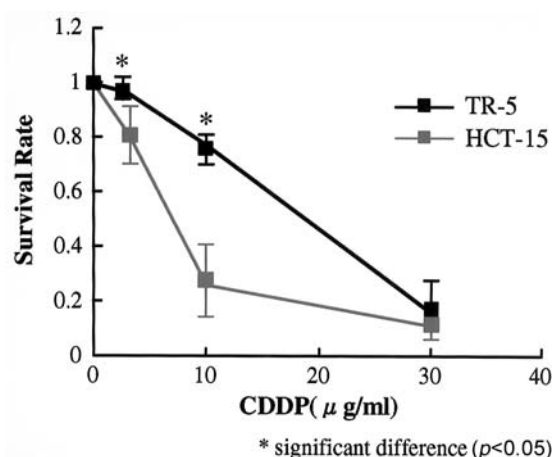


Figure 5. 3×10^3 HCT-15 or TR-5 cells/well were incubated in 96-well microplates, cultured with control medium plus FCS for 24 h and exposed to various concentrations of CDDP for 48 h before the end point. The viability and survival of cancer cells were evaluated using the 3-(4,5-dimethylthiazol-2y-l)-2,5-diphenyl tetrazolium bromide (MTT) assay. Statistically significant differences were observed at 3 and 10 µg/ml of CDDP between HCT-15 and TR-5 cells.

(ABC) superfamily of transport proteins and modifications in signal transduction pathways such as phosphatidylinositol 3'-kinase are associated with cisplatin resistance (25, 26). Investigations to gain a fuller understanding of the cellular and molecular mechanisms of cisplatin resistance in malignant cells are being actively carried out, with the expectation of future improved clinical outcomes based on a better understanding of these drug resistance mechanisms.

Multidrug resistance or heavy metal detoxification are closely related with various kinds of integral membrane proteins belonging to the ABC superfamily of transport proteins, including the 170 kDa P-glycoprotein MDR-1 and the 190 kDa MRPs (3). MRP-1 is an integral membrane phosphoglycoprotein capable of conferring resistance to multiple chemotherapeutic agents. Several laboratories have reported that overexpression of MRP-1 is associated with a reduction in intracellular cisplatin accumulation (23). MRP-1 is associated with drug resistance in a variety of tumor types including lung carcinoma (10-12), gastric carcinoma (13), retinoblastoma (14) and acute myeloid leukemia (15); however, some studies have reported lack of increased MRP-1 expression in CDDP-resistant cells (27, 28).

In the present study, we observed MRP-1 m-RNA overexpression and acquired resistance to CDDP resistance in conjunction with COX-2 overexpression in HCT-15 cells transfected by the COX-2 gene. We demonstrated re-sensitization to CDDP cytotoxicity in TR-5 cells following pretreatment by a COX-2 inhibitor. These findings suggest that MRP-1 may be a critical molecule responsible for the development of chemoresistance to CDDP associated with

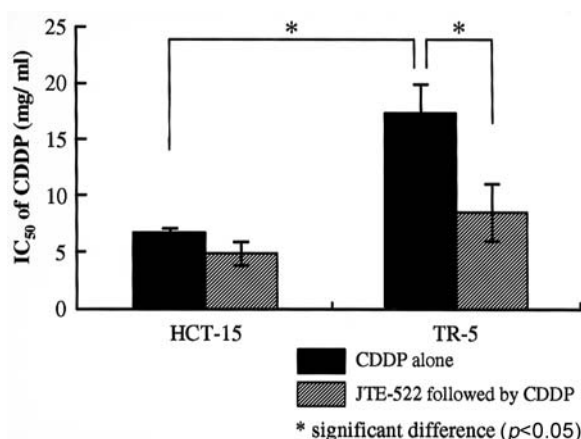


Figure 6. IC_{50} of CDDP alone and IC_{50} of JTE522 followed by CDDP were calculated from the concentration-survival curves for HCT-15 and TR-5. CDDP demonstrated a remarkable efficacy following TR-5 pre-treatment with JTE522 (as demonstrated by a decline in IC_{50} from 17.3 ± 2.6 mg/ml to 8.6 ± 2.5 mg/ml), while HCT pre-treatment with JTE522 caused only a slight decrease in the IC_{50} value of CDDP (from 6.7 ± 0.3 mg/ml to 4.9 ± 1.0 mg/ml).

experimental COX-2 gene overexpression in the HCT-15/TR-5 model. However, detailed studies of MRP subfamilies, including MRP-1, and comprehensive screening of all molecules connected with chemosensitivity to CDDP would be necessary to fully describe the mechanisms involved in the HCT-15/TR-5 model.

COX-2 has been found to be up-regulated under inflammatory conditions *in vitro* and *in vivo*. The anti-inflammatory properties of NSAIDs result from inhibition of COX-2, whereas the adverse effects of NSAIDs such as gastrointestinal damage and renal dysfunction appear to be associated with the inhibition of COX-1 (29). This difference in activity between COX-1 and COX-2 suggests a pharmacological usefulness for selective COX-2 inhibitor drugs in the management of inflammation.

Enhanced expression of COX-2, but not COX-1, has been found in various cancers. Recent studies suggest a relationship between COX-2-mediated PG biosynthesis and the development, proliferation and metastasis of various cancers. Several studies have reported a relationship between COX-2 expression and molecular modifications in several molecules, including VEGF, ABC superfamily of transport proteins (MDR or MRPs) (30-34). Such molecular modifications occurring during carcinogenesis in cells acquiring malignant potential might represent markers useful for assessing cancer therapy in experimental and clinical study.

We demonstrated that, following pre-treatment with the COX-2 selective inhibitor JTE-522, CDDP effectively suppressed cell growth in TR-5 cells (29). Previously we reported possible inhibition of cancer growth with JTE-522 alone or JTE-522 in combination with CDDP in a human

gastric cancer model both *in vitro* and *in vivo* (35). We previously showed that 100 μ M JTE-522 has an antitumor effect against gastric cancer cells and that JTE-522 could potentially be added to chemotherapeutic regimens including CDDP without increasing toxicity, since COX-2 selective inhibitors were designed to show anti-inflammatory activity without adverse effects as well as antitumor activity (36-38).

Conclusion

In the present study, we established the COX-2-overexpressed colon cancer cell line TR-5 from HCT-15 cells (which does not express COX-2 m-RNA). We evaluated chemosensitivity to CDDP in both TR-5 and HCT-15 cells, and we also quantified expressions of messenger RNA for both multidrug resistance gene 1 (MDR1) and multidrug resistance-associated protein 1 (MRP1), which are reported to be molecules related to CDDP resistance (10-15). In addition, we assessed the efficacy of the COX-2 selective inhibitor JTE522 in overcoming drug resistance to CDDP resulting from COX-2 gene induction in TR-5 cells. These findings suggest that the combination of JTE522 and CDDP might have clinical potency against colon cancer.

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Received March 12, 2004

Revised June 8, 2004

Accepted June 21, 2004