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ORIGINAL ARTICLE

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Combination chemotherapy of paclitaxel and cisplatin induces apoptosis with Bcl-2 phosphorylation in a cisplatin-resistant human epidermoid carcinoma cell line

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Abstract *Purpose*: Paclitaxel (Taxol, TXL) is an antimicrotubule agent that stabilizes microtubules, arrests the cell cycle at the G_2/M phase and induces apoptosis. In vitro drug sensitivity assays have shown that the combination of TXL and CDDP is more effective in CDDP-resistant ovarian carcinoma cell lines, with different cytotoxicities depending on the sequence of drug exposure. CDDP also shows poor results in human epidermoid carcinoma particularly of the head and neck region. *Methods*: We investigated the effects and the molecular mechanisms of combination chemotherapy with TXL and CDDP in the CDDP-resistant cell line A431/CDDP2, and in its parental human epidermoid cell line A431/P. Drug sensitivity was determined using the MTS assay and cell cycle perturbation was analyzed using flow cytometry. DNA fragmentation was then analyzed and the protein levels of caspase-3 and Bcl-2, and phosphorylated of Bcl-2 were determined by Western blotting. *Results*: In the drug sensitivity assay, exposure to CDDP prior to TXL was more effective than exposure TXL prior to CDDP in A431/P cells. In A431/CDDP2 cells, exposure to TXL prior to CDDP was more effective than exposure to CDDP prior to TXL. Exposure to TXL arrested the cells in the G_2/M phase in both cell lines. In A431/CDDP2 cells, exposure to TXL prior to CDDP arrested the cells in the G_2/M phase, an effect caused by either CDDP or TXL. Analysis of DNA fragmentation showed similar results to the drug sensitivity assay. Expression of caspase-3 protein active form was detected following exposure to TXL only and to the TXL/CDDP combination in both A431/P and A431/CDDP2 cells, but phosphorylation of Bcl-2 protein was detected only following exposure to TXL and only in A431/CDDP2 cells. *Conclusions*: These results indicate that exposure to TXL prior to CDDP plays a key role in circumventing CDDP resistance by phosphorylating Bcl-2 protein in the human epidermoid carcinoma cell line A431/CDDP2.

Keywords Cisplatin (CDDP) · Paclitaxel (TXL) · Apoptosis · Cisplatin-resistant · Bcl-2 phosphorylation

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Introduction

Anticancer chemotherapy is one of the most effective treatments for malignant neoplasms. Cisplatin [*cis*-diamminedichloroplatinum(II), CDDP] is one of the most widely used anticancer drugs [1]. It used as a base for single or combination chemotherapy and as a radiosensitizer in the treatment of solid tumors including squamous cell carcinoma of the head and neck region [2, 3, 4]. The major cytotoxic action of CDDP is considered to be platination of DNA which leads to the induction of interstrand and predominantly intrastrand crosslinks, inducing apoptosis [5]. However, one of the major obstacles to success in anticancer chemotherapy is the development of anticancer drug resistance in cancer cells. The molecular mechanisms of CDDP resistance have been shown to include decreased intracellular drug accumulation [6], increased intracellular glutathione and metallothioneins [7, 8], reduction in DNA crosslinking as a consequence of decreased accessibility of DNA to the drug, and/or increased DNA repair [9]. Reversing drug resistance would be a most important approach to improving the success of anticancer chemotherapy.

Paclitaxel (Taxol, TXL) is an antimicrotubule agent isolated from the western yew tree *Taxus brevifolia* [10, 11]. This agent has demonstrated clinical efficacy in the treatment of ovarian cancer, non-small-cell cancer,

breast cancer [12], and head and neck cancer [13]. It binds to the β-subunit of tubulin, accelerating the polymerization of tubulin [14]. Consequently, cells are arrested at the G_2/M phase of the cell cycle [15], and apoptosis is induced [16]. Moreover, TXL downregulates the expression of Bcl-X_L [17], upregulates Bax [18], and causes the phosphorylation of Bcl-2 [19], which is generally believed to initiate apoptosis. TXL is activated by MAPK pathways such as the ERK and JNK/SAPK signaling pathways [20]. CDDP and TXL have been used clinically in combination chemotherapy and have been examined in several clinical trials in ovarian, lung, breast, head and neck, cervical, and gastrointestinal carcinomas [21]. The interaction between TXL and CDDP has mainly been examined in vitro in ovarian carcinoma cell lines, and there is evidence of synergistic or antagonistic effects depending on the schedule of the TXL/CDDP combination therapy [22, 23]. However, mechanism of the interaction has not been clarified in several cell lines.

In the present study, we investigated the effects and interaction mechanisms of TXL/CDDP combination chemotherapy in the human epidermoid carcinoma cell line A431 and its CDDP-resistant subline A431/CDDP2 [24], and explored the possibility of circumventing CDDP resistance in human epidermoid carcinomas.

Materials and methods

Anticancer drugs

CDDP was purchased from Nippon Kayaku (Tokyo, Japan). TXL was purchased from Sigma Chemical Company (St. Louis, Mo.).

Cell lines

We used the CDDP-resistant subline A431/CDDP2 derived from the previously established human epidermoid carcinoma cell line A431 [24]. Cells were cultured in DMEM/F-12 (Life Technologies, Rockville, Md.) containing 5% fetal bovine serum (Life Technologies) and 1% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO_2.

Drug sensitivity assay

Cell survival was determined by a CellTiter 96 aqueous nonradioactive proliferation assay (Promega Corporation, Madison, Wis.). Cells in exponential growth were washed with phosphate-buffered saline (PBS), trypsinized with 0.25% trypsin/EDTA for 5 min at 37°C, counted and seeded as a single-cell suspension at a density of 5000 cells/well in 96-well microtiter plates (Becton Dickinson, Franklin Lakes, N.J.). After 24 h, various concentrations of CDDP or TXL were added to the medium, CDDP for 5 h and TXL for 24 h. After drug exposure, the drug-containing medium was aspirated from the plate, fresh medium was added and the cultures incubated for 3 days. Control dishes without anticancer drugs were treated identically. After incubation, plates were washed with PBS and 20 µl MTS/PMS solution was pipetted into each well of the 96-well microtiter plate. These were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 and the absorbance values recorded at 490 nm using a microplate reader (Bio-Rad Laboratories, Hercules, Calif.). The drug concentrations inhibiting cell growth by 50% (IC_{50}) were obtained by graphical analysis.

Four different protocols were used to investigate the interaction of CDDP and TXL:

- Protocol 1: exposure to CDDP for 5 h
- Protocol 2: exposure to CDDP for 5 h prior to incubation in drug-free medium for 24 h and finally exposure to TXL for 24 h
- Protocol 3: exposure to TXL for 24 h
- Protocol 4: exposure to TXL for 24 h prior to incubation in drug-free medium for 24 h and finally exposure to CDDP for 5 h

At the end of treatment, cells were washed with PBS and incubated at 37°C in an atmosphere containing 5% CO_2 for 72 h. Cell survival ratios were determined as described above. CDDP and TXL were used at their IC_{50} values.

Cell cycle analysis by flow cytometry

A431/P and A431/CDDP2 cells were cultured at 1×10^6 cells per 60-mm dish. The same protocols as described for the drug sensitivity assay were used. The cells were then incubated in drug-free medium for 0, 12 and 24 h. After incubation, the cells were harvested by trypsinization and fixed in 70% ethanol. Following treatment with RNase (1 mg/ml in 0.1 *M* phosphate buffer, pH 7.0; Takara, Tokyo, Japan), the cells were stained with propidium iodide solution (50 mg/ml in 0.1% sodium citrate, 0.1% NP-40) and then analyzed using a flow cytometer (EPICS XL; Beckman Coulter, Hialeah, Fl.) and the cell cycle distribution was calculated using the MULTICYCLE program (Beckman Coulter).

DNA fragmentation ELISA

The release of fragmented DNA into the cytoplasm during apoptotic cell death was measured using a cellular DNA fragmentation ELISA kit (Boehringer Mannheim, Maylan, France). In brief, exponentially growing A431/P and A431/CDDP2 cells were labeled with 10 µ*M* BrdU for 18 h at 37°C in a humidified atmosphere. After labeling, the cells were resuspended in culture medium and adjusted to 1×10^5 cells/ml, and 100 µl/well was transferred to a 96-well microtiter plate after the cells had been treated overnight with CDDP and/or TXL using the protocols described above. After incubation in drug-free medium for 72 h, the cells in the microtiter plate were lysed for 30 min at room temperature. The microtiter plate was centrifuged at 250 *g* for 10 min, and the supernatant was collected as the sample for assay. The samples were transferred to each well of a precoated microtiter plate and incubated for 90 min at room temperature. After washing, the samples were denatured and fixed by microwave irradiation (650 W) for 5 min. After cooling the plate for 10 min at −20°C, anti-BrdU peroxidase conjugate solution was added followed by incubation for an additional 90 min at room temperature. After washing, immunocomplexed anti-BrdU peroxidase was detected and measured at 450 nm using a microplate reader (Bio-Rad).

Western blotting

A431/P and A431/CDDP2 cells were cultured at 1×10^6 cells per 90-mm dish. The cells were treated using the protocols described above. After drug exposure, the cells were cultured in a drug-free medium for 24 h. Cells were lysed in lysis buffer (50 m*M* Tris,

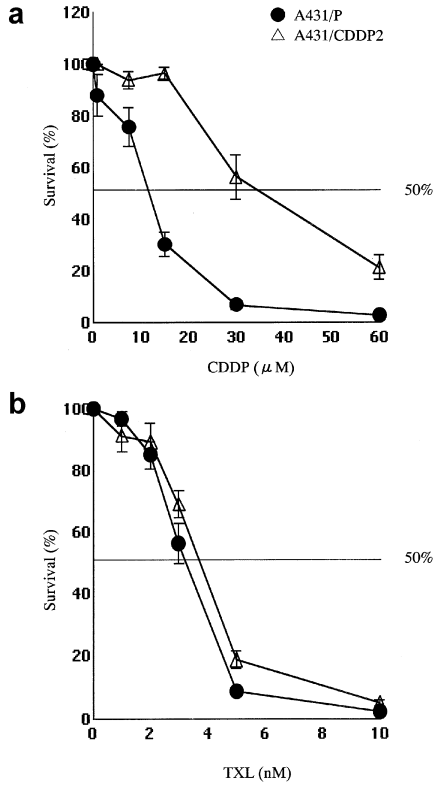

Fig. 1a, b Dose response curves for (**a**) CDDP and (**b**) TXL (● A431/P, △ A431/CDDP2). The points and bars represent the means ± SD of three determinations

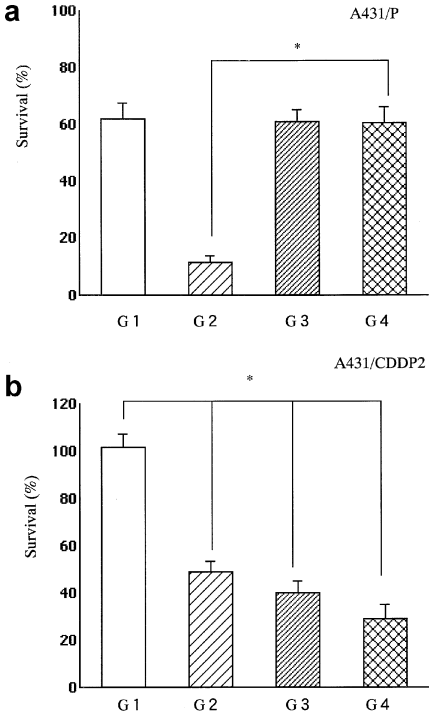

Fig. 2a, b Drug sensitivity MTS assays in (**a**) A431/P and (**b**) A431/CDDP2 cells (*G1–G4* drug exposure protocols 1–4 as described in Materials and methods). Each value is the mean ± SD of three determinations

150 m*M* NaCl, 1% NP40, 0.5% DOC, 7.5 μg/ml aprotinin, 50 μg/ml leupeptin, 1 m*M* PMSF, 1 m*M* sodium orthovanadate) and centrifuged at 10,000 *g* for 10 min at 4°C, and the amount of protein was determined using a DC protein assay (Bio-Rad). The lysates were boiled for 5 min, separated by 15% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.) in transblotting buffer (25 m*M* Tris, 192 m*M* glycine, 20% v/v methanol, pH 8.3). The membranes were incubated for 1 h with a blocking buffer (5% non-fat milk, 0.1% Tween 20 in TBS) and then with monoclonal antibodies to mouse anti-Bcl-2 (BD Biosciences, San Jose, Calif.), anti-phospho-Bcl-2 (Cell Signaling Technology, Beverley, Mass.), polyclonal antibodies to rabbit anti-caspase-3 (BD Biosciences) diluted 1:1000 in the same buffer for 16 h, washed three times with TBS-T, and incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Biosciences, Piscataway, N.J.; diluted 1:1000 in TBS-T) for 1 h. After the membranes had been washed three times with TBS-T, the signal was visualized by enhanced chemiluminescence with an ECL system (Amersham Biosciences).

Results

Drug sensitivity assay

The sensitivities of A431/P and A431/CDDP2 cells to a 5-h exposure to CDDP were markedly different (IC_{50} values 11.6 μ*M* and 34.8 μ*M*, respectively; Fig. 1a). The effects of a 24-h exposure to TXL were similar in the two cell lines (IC_{50} 3.3 n*M* in A431/P cells and 3.8 n*M* in A431/CDDP2 cells; Fig. 1b). Figure 2 shows the results of drug sensitivity assay with protocols 1, 2, 3 and 4 using the drugs at their IC_{50} values. In A431/P cells, protocol 2 was more effective than protocol 4, and protocol 4 had a similar effect to protocol 3 (Fig. 2a). Conversely, in A431/CDDP2 cells, protocol 4 was more effective than protocol 2 (Fig. 2b). These results show that the CDDP-resistant cell line A431/CDDP2 had quite the reverse drug sensitivity to CDDP and TXL than the parent cell line A431/P.

Effect of drug treatment on cell cycle progression

Using the same protocols used for the drug sensitivity assay, we determined the cell cycle change in these cell lines by flow cytometry. A431/P cells treated with protocols 1 and 4 were blocked at the G_2/M phase after a 24-h drug exposure, but with protocol 2 the proportions of cells in the G_1 and G_2/M phases were immediately reduced (Fig. 3a). On the other hand, A431/CDDP2 cells treated with protocols 1 and 2 showed similar non-significant cell cycle changes after a 24-h drug exposure, but those treated with protocols 3 and 4 were clearly blocked at the G_2/M phase after a 24-h drug exposure (Fig. 3b). In these cell lines, CDDP only and CDDP prior to TXL caused different cell cycle perturbations, but TXL only and TXL prior to CDDP caused similar cell cycle perturbations, blocking the cells in the G_2/M phase after a 24-h drug exposure.

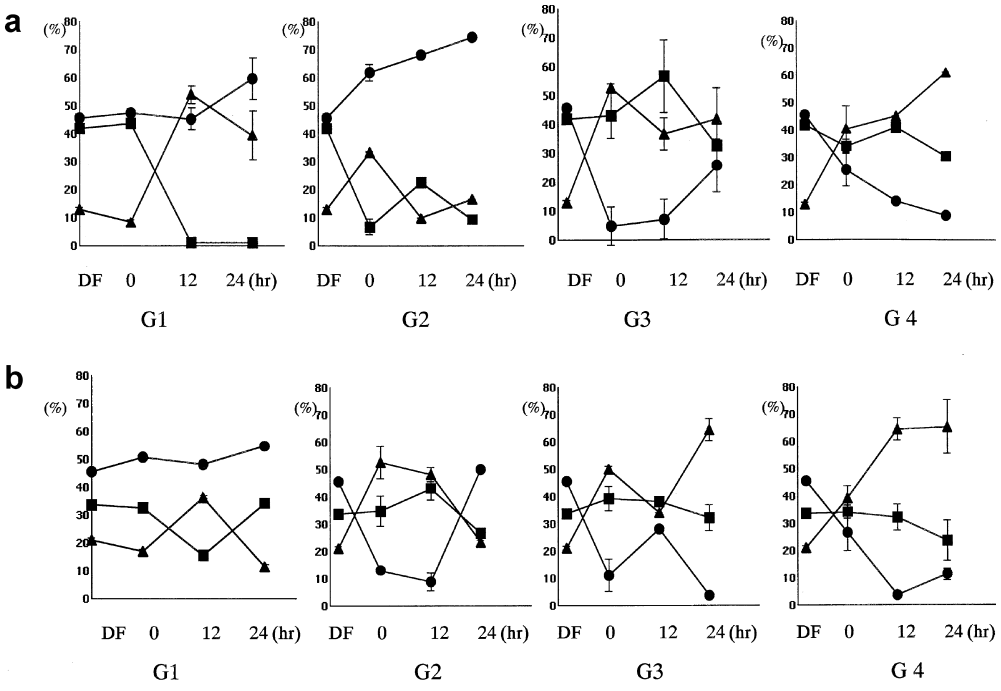

Fig. 3a, b Time courses of cell cycle perturbation in (**a**) A431/P and (**b**) A431/CDDP2 cells (*G1–G4* drug exposure protocols 1–4 as described in Materials and methods; *DF* drug-free condition; ■ G_1 phase, ● S phase, ▲ G_2/M phase). Points and bars represent the means ± SD of three determinations

DNA fragmentation assay

To quantify the apoptotic response to the four different drug exposure protocols in the two cell lines, we used an ELISA method that allowed the specific detection and quantification of BrdU-labeled DNA fragments. Quantitative analysis of DNA fragmentation is shown in Fig. 4. In A431/P cells, the amount of DNA fragmentation was higher in cells treated with protocol 2 than in those treated with protocol 4 (Fig. 4a). In A431/CDDP2 cells, the amount of DNA fragmentation was higher in cells treated with protocol 4 than the inverse protocol (Fig. 4b). This result indicates that exposure to TXL prior to CDDP induced apoptosis to a greater extent than exposure to CDDP prior to TXL in the CDDP-resistant cell line.

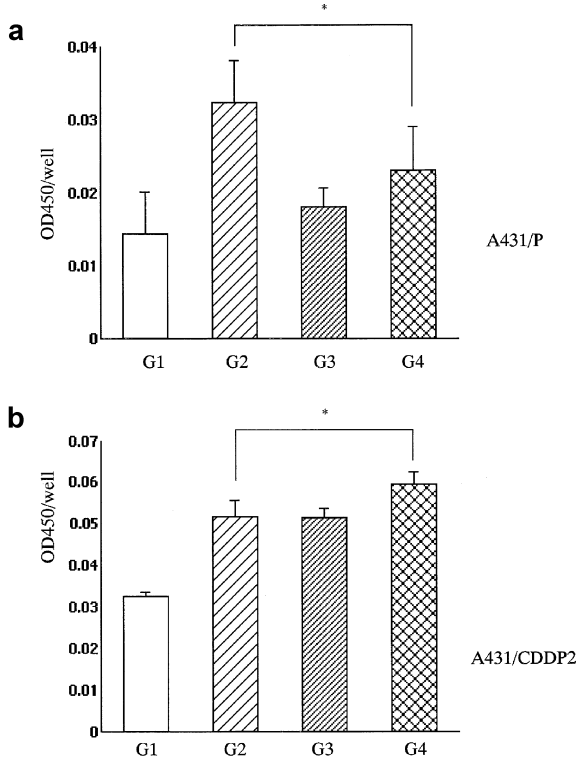

Fig. 4a, b DNA fragmentation assays in (**a**) A431/P and (**b**) A431/CDDP2 cells (*G1–G4* drug exposure protocols 1–4 as described in Materials and methods). Each value is the mean ± SD of three determinations

Detection of caspase-3 and Bcl-2 phosphorylation

Protein expression of caspase-3 and Bcl-2 as determined by Western blotting is shown in Fig. 5. Although procaspase-3 expression was detected in the cell lines, the caspase-3 active form (17 kDa) was detected in A431/P after a 48-h drug exposure. The expression level of caspase-3 active form in A431/P cells was higher in cells exposed to CDDP prior to TXL (protocol 2) than in those exposed to TXL prior to CDDP (protocol 4). In A431/CDDP2 cells, the active form was also detected after a 48-h drug exposure. Caspase-3 active form was at almost the same level in cells exposed to TXL prior to CDDP (protocol 4). Bcl-2 protein expression was detected A431/CDDP2 cells in all drug exposure protocols, but not detected A431/P cells. In A431/CDDP2 cells, phospho-Bcl-2 expression was only detected in cells exposed to TXL (protocol 3).

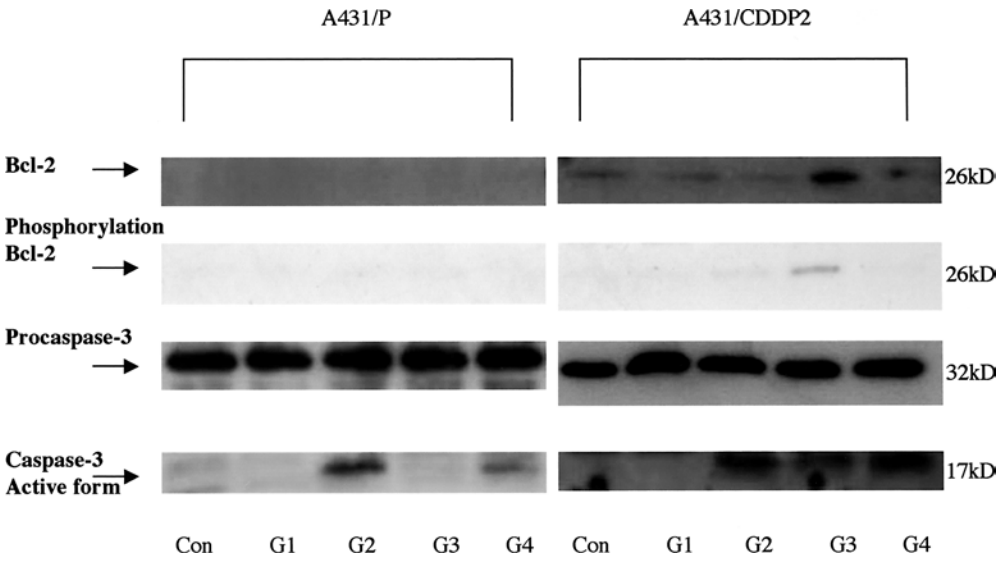

Fig. 5 Detection of procaspase-3, caspase-3 active form, Bcl-2, and phosphorylated Bcl-2 proteins in A431/P and A431/CDDP2 cells by Western blotting (*G1–G4* drug exposure protocols 1–4 as described in Materials and methods; *Con* control)

Discussion

CDDP is accepted as the most effective treatment for squamous cell carcinoma of the head and neck cancer. However, in cancer patients who have been previously treated with CDDP, the effect of CDDP treatment often decreases, and the tumors frequently acquire CDDP resistance. Therefore, we need a second-line chemotherapy regimen that uses other anticancer agents to counteract CDDP resistance. In the present study, we investigated the effects of TXL, alone or in combination with CDDP, in the human epidermoid carcinoma cell line A431/P and its CDDP-resistant cell line A431/CDDP2.

TXL has a different mechanism in cancer cells that have accumulated in the G_2/M phase of the cell cycle and in which apoptosis has been induced. In clinical trials, CDDP and TXL combination chemotherapy has been evaluated in patients with various cancers, such as lung, breast, ovarian and head and neck. In vitro, the mechanism of action of CDDP and TXL combination chemotherapy in cancer cells has mainly been investigated in ovarian cell lines [22]. In a CDDP-resistant cell line, the effect of TXL has been shown to be inhibited by exposure to CDDP prior to TXL [23]. In the present study, exposure of A431/P cells to TXL prior to CDDP showed a poor effect in comparison with exposure to TXL only. Exposure to CDDP prior to TXL was more effective than exposure to CDDP or TXL only. Conversely, in the CDDP-resistant subline A431/CDDP2, exposure to TXL prior to CDDP was more effective than exposure to TXL only or CDDP prior to TXL. This result suggests that exposure to TXL prior to CDDP circumvented CDDP resistance in the CDDP-resistant cell line A431/CDDP2. Another group has reported that, in ovarian cancer cell lines, exposure to TXL prior to CDDP has a synergistic effect in a CDDP-resistant cell line, but an antagonistic effect in a CDDP-sensitive cell line [22]. Our results were similar, in that exposure TXL prior to CDDP had a synergistic effect in the CDDP-resistant cell line and an antagonistic effect in the CDDP-sensitive cell.

TXL induces cell cycle arrest at the G_2/M phase [15] and cell death [16]. Our flow cytometry results indicated that exposure to TXL only or TXL prior to CDDP increased the proportion of cells in the G_2/M phase 24 h after final drug treatment. In A431/CDDP2 cells exposed to TXL prior to CDDP at 0 h after treatment were not clearly arrested at the G_2/M phase. From 12 to 24 h after CDDP exposure, A431/CDDP2 cells were clearly arrested at the G_2/M phase. These findings indicate that accumulation at the G_2/M phase following exposure to TXL prior to CDDP might be an effect of TXL or an effect of CDDP. CDDP also induces arrest of cells in the G_2/M phase in a similar manner to TXL [25]. The cell cycle perturbation in A431/CDDP2 cells following exposure to TXL prior to CDDP was similar to that in CDDP-sensitive A431/P cells following exposure to CDDP only.

We also quantified DNA fragmentation caused by apoptosis to determine the cytotoxic activity of the anticancer agents. The major cytotoxic action of CDDP is considered to be platination of DNA which leads to induction of interstrand and predominantly intrastrand cross-links, and apoptosis [5]. The major cytotoxic action of TXL is considered to be via binding to the β-subunit of tubulin, accelerating the polymerization of tubulin. In addition, cancer cells are arrested at the G_2/M phase of the cell cycle, and undergo apoptosis. We compared the apoptotic responses of A431/P and A431/CDDP2 cells. In A431/CDDP2 cells, quantitative DNA fragmentation showed a greater increase following exposure to TXL prior to CDDP than following exposure to CDDP prior to TXL. This finding was correlated with the results of the drug sensitivity assay. These results indicate that the increased cytotoxicity of the TXL

and CDDP combination was a result of an increase in apoptosis. The TXL and CDDP combination has also been shown to increase apoptosis in ovarian cancer cell lines in vitro [22]. We conjecture that different cytotoxicities in the same cell line following different drug exposure sequences were caused by different apoptosis-inducing mechanisms.

Cysteine proteases encoded by *Caenorhabditis elegans* ced-3 [26] and its mammalian homologue representing the ICE family, including caspase-3, appear to initiate key events in apoptosis and may be effectors of apoptotic cell death [27, 28]. Caspase-3 normally exists as an inactive proenzyme which, immediately prior to apoptotic cell death, is cleaved to a heterodimer comprising M_r 17,000 and 12,000 subunits that constitute the active protease [29]. Bcl-2 has the ability to exert a survival function in response to a wide range of apoptotic stimuli by blocking mitochondrial release of cytochrome *c* [30]. Bcl-2 is also phosphorylated following exposure to chemotherapeutic taxanes such as TXL and docetaxel (Taxotere) which promote microtubule assembly [31]. This phosphorylation inactivates the antiapoptotic function of Bcl-2 [32]. In this study, the caspase-3 active form was detected in CDDP-resistant A431/CDDP2 cells exposed to TXL only and in those exposed to TXL-CDDP in combination, and phosphorylated Bcl-2 was detected in cells exposed to TXL only. We have reported previously that Bcl-2 protein levels are higher in A431/CDDP2 cells than in CDDP-sensitive A431/P cells [33]. In our present study, Bcl-2 was phosphorylated by TXL in A431/CDDP2 cells. This result suggests that inhibition of the antiapoptotic function of Bcl-2 by phosphorylation by TXL plays a key role in the cytotoxicity of CDDP in A431/CDDP2 cells.

In conclusion, we found that exposure to TXL prior to CDDP was more effective in CDDP-resistant human epidermoid A431/CDDP2 cells and suggest that Bcl-2 phosphorylation by TXL is one of the most important effects circumventing CDDP resistance in human epidermoid carcinoma. Additional extensive studies are needed to elucidate in more detail the molecular mechanisms of TXL and CDDP combination chemotherapy to establish an effective clinical chemotherapy in human epidermoid neoplasms.

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