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ORIGINAL ARTICLE

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Activity of docetaxel in paclitaxel-resistant ovarian cancer cells

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Abstract *Purpose*: The aim of this study was to determine the behavior of docetaxel (DTX) in ovarian cancer cells resistant to paclitaxel (PTX). *Methods*: We used human ovarian adenocarcinoma cell lines KF, KFTx (PTX-resistant KF), SK-OV-3, and HAC-2. The sensitivity of the cells to PTX or DTX was determined by the MTT assay. Cellular accumulation of PTX and DTX was measured by high-performance liquid chromatography. mRNA of MDR-1 was detected by RT-PCR. Cell cycle distribution was determined by flow cytometry after exposure to the IC_{50} of each drug. Bcl-2 phosphorylation was determined by Western blot analysis. Activity for tubulin polymerization of each drug was examined by a β-tubulin polymerization assay. *Results*: KFTx cells had a 5.5-fold greater resistance to PTX and a 7.3-fold greater resistance to DTX than KF cells, indicating that KFTx cells had acquired cross-resistance to DTX. SK-OV-3 cells were sensitive and HAC-2 cells were resistant to both PTX and DTX. The gene expression of MDR-1 increased after exposure to DTX in KF and KFTx cells. Residual cellular accumulation of PTX and DTX was significantly lower in KFTx cells than in KF cells. In contrast, MDR-1 expression was not detected in SK-OV-3 and HAC-2 cells. Flow cytometric analysis indicated no differences in alterations of cell cycle distribution following exposure to the two drugs. Bcl-2 phosphorylation occurred after exposure to DTX at a concentration equivalent to the clinical dose, but did not occur after exposure to PTX in KFTx cells. In HAC-2 cells, Bcl-2 phosphorylation was not detected after exposure to DTX or PTX at concentrations equivalent to the clinical doses. DTX showed greater tubulin polymerization activity than PTX in KFTx cells. β-tubulin polymerization did not correlate with the concentration of PTX or DTX, suggesting that alteration in the tubulin reaction might contribute to the resistance in HAC-2 cells. *Conclusions*: The present study suggests that the mechanisms involved in cytotoxicity of and resistance to PTX and DTX do not differ, but DTX has a greater cytotoxic potential in PTX-resistant cells with an efflux system.

Keywords Paclitaxel · Docetaxel · Bcl-2 phosphorylation · MDR-1 gene · Polymerized tubulin

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Introduction

Paclitaxel (PTX) is an active anticancer agent used to treat a broad spectrum of tumors, including ovarian cancer [8]. As a promoter of tubulin polymerization, PTX changes the dynamic equilibrium between assembly and disassembly of microtubules, disrupts the formation of the normal spindle at metaphase, and causes the blockade of mitosis at the G_2M phase [13, 20]. The oncogenic protein, Bcl-2, functions as a potent inhibitor of apoptosis. Cytotoxicity following DNA damage is influenced by the Bcl-2 phosphorylation status. Recent studies indicate that disruption of microtubular architecture induces Raf-1 activation and Bcl-2 phosphorylation [3, 22, 27, 28]. The interaction of PTX with tubulin and microtubules is essential for Bcl-2 phosphorylation and apoptosis [1, 4]. Due to its hydrophobic nature, resistance to PTX is associated with the induction of the multidrug resistance gene (MDR-1) encoding p-glycoprotein [20]. We have also found that resistance to PTX is related to decreased cellular accumulation of PTX and expression of the MDR-1 gene [15, 16].

Docetaxel (DTX), the second taxoid derivative, has greater cytotoxic potency than PTX [10, 12, 21, 23]. Recent clinical studies have shown that DTX is an effective chemotherapeutic agent in patients with

ovarian cancer who have previously been treated without success with PTX-based chemotherapy, suggesting an apparent lack of cross-resistance between the two taxane compounds [2, 17, 25]. This phenomenon may be explained by differences in the mechanism of action or in other pharmacological properties. However, the mechanism of DTX efficacy in PTX-resistant cells has not been clarified.

In the present study we investigated the behavior of DTX in PTX-resistant ovarian cancer cells.

Materials and methods

Cell lines and culture

We used human ovarian adenocarcinoma cell lines as follows. The KF cell line was kindly provided by Prof. Kikuchi, National Defense Medical College. A PTX-resistant cell line (KFTx) was established by continuous exposure of KF cells to stepwise escalating concentrations of PTX for over 18 months. The SK-OV-3 cell line was obtained from the American Type Culture Collection. The HAC-2 cell line was kindly provided by Dr. Nishida, Tsukuba University. These cell lines were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37°C.

Sensitivity to PTX or DTX

The sensitivity of the cells to PTX or DTX was determined by the MTT assay [19]. Briefly, cells were diluted with culture medium to the seeding density (10^5 cells/ml), suspended in 96-well tissue culture plates (100 μl/well) (Sumitomo Bakelite, Tokyo, Japan), and preincubated at 37°C for 4 h. Cells were then treated for 24 h with 20 μl of various concentrations of PTX (0.1–16 μ*M*) or DTX (0.03–5.0 μ*M*) to obtain a dose-response curve for each agent. PTX and DTX were dissolved in 100% dimethylsulfoxide (DMSO) to 1 m*M* and in 100% ethanol to 2 m*M*, respectively. The solutions were diluted to the required concentrations in culture medium as needed. The final concentrations of DMSO and ethanol were $<0.1\%$ and were found to be nontoxic to the cell lines. After incubation, 20 μl MTT solution (2.5 mg/ml) was added to each well and the plates were further incubated for 4 h. DMSO (100 μl) was added to each well to solubilize the MTT-formazan product after removal of the medium. Absorbance at 570 nm was measured with a microplate reader model 450 (BIO-RAD, Richmond, Calif.). Dose-response curves were plotted on a semi-log scale as percentage of the cell numbers in control cultures not exposed to drug.

Analysis of cell cycle distribution

Cell cycle distribution was determined by flow cytometry 6, 12 and 24 h after exposure to each drug at its IC_{50}. For flow cytometric analysis, cells (2×10^6) were trypsinized, collected by centrifugation, fixed in 70% ethanol at 4°C for 1 h, and then resuspended in phosphate-buffered saline (PBS) containing 40 μg/ml propidium iodide and 0.1 mg/ml RNase. After 30 min at 37°C, the cells were analyzed with a FACstar cytofluorimeter (Becton Dickinson, San Jose, CA).

mRNA expression of MDR-1

Cells were exposed to PTX or DTX at their IC_{50} values for 1, 2, 4 and 8 h and then collected. Total RNA was isolated from each sample by the acid guanidinium thiocyanate-phenol-chloroform extraction method [5]. mRNA of MDR-1 was detected by reverse transcription-polymerase chain reaction (RT-PCR) using a GeneAmp RNA PCR Core Kit (Perkin-Elmer Corporation, Norwalk, Conn.). Briefly, cDNA was synthesized from the isolated RNA by reverse transcription of RNA. PCR amplification was carried out in 25 μmol/l of a magnesium chloride solution (2 μmol/l), 10× polymerase chain reaction buffer II (1×), distilled water (65.5 μl), and AmpliTaq deoxyribonucleic acid polymerase (2.5 U/100 ml, Perkin-Elmer). Samples were amplified for 35 cycles of denaturation (30 s at 95°C), annealing (45 s at 58°C), synthesis (90 s at 72°C), and primer extension (7 min at 72°C). The PCR primers used were 5′-ACACCCGACTTACAGAT-GATGTCTC-3′ (forward) and 5′-CGAGATGGGTAACTGAAGTGAA-CAT-3′ (reverse) and the expected product size was 623 bp. PCR products were resolved on 2% agarose gel with Marker IV (Wako, Osaka, Japan).

Residual cellular accumulation of PTX and DTX

KF or KFTx cells were exposed to PTX or DTX at their IC_{50} values for 2 h and were incubated in drug-free medium for 1 or 2 h. After rinsing with PBS, cell suspensions were centrifuged at 150 *g* for 10 min. Samples were sonicated in PBS on ice. PTX and DTX was quantified by reverse-phase high-performance liquid chromatography according to the method of Longnecker et al. [18]. The residual rate of each drug was calculated from the formula: residual rate (%) = 100×cellular drug concentration/cellular concentration at the end of exposure to drug.

Bcl-2 phosphorylation

Bcl-2 phosphorylation was determined by Western blot analysis. Cells were exposed to PTX or DTX at their IC_{50} values for 6, 12, 24 and 48 h, and were solubilized on ice in lysis buffer (50 m*M* Tris-HCl, 125 m*M* NaCl, 0.1% NP40, 5 m*M* ethylenediamine tetraacetic acid, 50 m*M* NaF, 0.1% phenylmethyl sulfonyl fluoride, and protease inhibitor) and centrifuged at 25,000 *g* for 30 min. The total protein concentration in the supernatant was measured, and samples of 60 μg protein were separated by electrophoresis on a 12% polyacrylamide gel.

The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) and were visualized with antimouse IgG coupled to horseradish peroxidase, using enhanced chemiluminescence according to the manufacturer's recommendations. The anti-Bcl-2 antibodies were purchased from DAKO (Glostrup, Denmark). Additionally, after exposure to PTX or DTX at concentrations calculated to be equivalent to standard clinical doses (175 mg/m^2 of PTX and 70 mg/m^2 of DTX) for 12 h, Bcl-2 phosphorylation was determined using the same procedure [7, 14].

β-Tubulin polymerization assay

β-Tubulin polymerization was quantified using a simple assay modified from that of Blagosklonny et al. [3]. Based on the results of Bcl-2 phosphorylation, cells were treated with PTX or DTX for 12 h as follows: 25.4–651 n*M* PTX and 0.95–25 n*M* DTX for KF cells, 500–2500 n*M* PTX and 120–500 n*M* PTX for KFTx cells, 11–126 n*M* PTX and 10–50 n*M* DTX for SK-OV-3 cells, and 1200–12000 n*M* PTX and 600–6000 n*M* PTX for HAC-2 cells. The cells were then washed twice with PBS, lysed for 5 min with 100 μl hypotonic buffer (1 m*M* $MgCl_2$, 2 m*M* EGTA, 0.5% NP40, 2 m*M* phenylmethylsulfonyl fluoride, 200 U/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 5 m*M* ϵ-amino-caproic acid, 0.01 n*M* benzamidine and 20 m*M* Tris-HCl, pH 6.8). The cells were rinsed with an additional 100 μl, and this was pooled with the lysates. The samples were centrifuged at 14,000 rpm for 10 min at room temperature, and the supernatants containing soluble (cytosolic)

tubulin were separated from the pellets containing polymerized (cytoskeletal) tubulin. The pellets were resuspended in 200 µl hypotonic buffer after sonication. Each fraction was mixed with 70 µl 4× SDS-PAGE sample buffer (45% glycerol, 20% β-mercaptoethanol, 9.2% SDS, 0.04% bromophenol blue and 0.3 *M* Tris-HCl, pH 6.8), heated for 6 min at 95°C, and analyzed by 12% polyacrylamide gel electrophoresis. After transfer onto a PVDF membrane, immunoblotting was performed using a primary anti-β-tubulin antibody (Sigma Chemical Company, St. Louis, Mo.).

Expression levels of soluble tubulin and polymerized tubulin were quantified using the public domain NIH image program (written by W. Rasband, US NIH, and available at zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, USA, part number PB93-504648). The proportions of polymerized tubulin after exposure to each concentration were measured, and then dose-response curves were plotted on a semi-log scale.

Statistical analysis

All assays were performed in triplicate. Statistical analyses were performed using the Statview version 4.5-J program (Hulinks, Tokyo Japan). The results of the β-tubulin polymerization assay were subjected to analysis of covariance (ANCOVA). P values <0.05 were considered statistically significant.

Results

KFTx cells showed a 5.5-fold greater resistance to PTX than KF cells. On the other hand, the IC_{50} value of DTX in KFTx cells was 7.3-fold greater than that in KF cells, indicating that KFTx cells had acquired cross-resistance to DTX. The IC_{50} values revealed that SK-OV-3 cells were sensitive and HAC-2 cells were resistant to both PTX and DTX (Table 1).

MDR-1 mRNA was not detected in SK-OV-3 or HAC-2 cells. The MDR-1 gene appeared in KF and KFTx cells, but the expression was weak in KF cells. Expression of MDR-1 2 h after exposure to DTX increased in both KF and KFTx cells (Fig. 1). Residual cellular accumulation levels of PTX at the end of exposure were 8.9 ng/10^6 cells in KF cells and 3.5 ng/10^6 cells in KFTx cells. Equivalent values for DTX were 4.1 ng/10^6 cells and 1.8 ng/10^6 cells, respectively. The residual rates of PTX and DTX were significantly lower in KFTx cells than in KF cells over 1 h after exposure to each agent (Fig. 2).

Flow cytometric analysis showed accumulation of cells in pre-G_1 6 h after exposure to PTX or DTX and in G_2M phase 12 h after exposure. The alterations in cell cycle distribution did not differ between these two agents in any of the four cell lines (Fig. 3). Bcl-2 phosphorylation occurred over 12 h after exposure to PTX or DTX at their IC_{50} values in all cell lines (Fig. 4A, B), suggesting that PTX and DTX induce apoptosis through the same pathway. Bcl-2 phosphorylation occurred in KF and SK-OV-3 cells after exposed to PTX and DTX at concentrations equivalent to their clinical doses (268 n*M* PTX and 52 n*M* DTX; Fig. 4C). When KFTx cells were exposed to PTX or DTX at concentrations equivalent to their clinical doses, Bcl-2 phosphorylation occurred with DTX, but not with PTX. In HAC-2 cells, Bcl-2 phosphorylation was not observed after exposure to DTX or PTX at clinical doses.

The β-tubulin polymerization assay showed that the slope of the regression line did not differ between PTX and DTX in KF and SK-OV-3 cells. In contrast, the slope for PTX significantly decreased but that for DTX did not in KFTx cells, indicating that PTX had significantly lower tubulin polymerization activity in KFTx cells. In HAC-2 cells, β-tubulin polymerization was not significantly correlated with the concentration of PTX or DTX, suggesting that tubulin reaction might contribute to resistance in HAC-2 cells (Fig. 5).

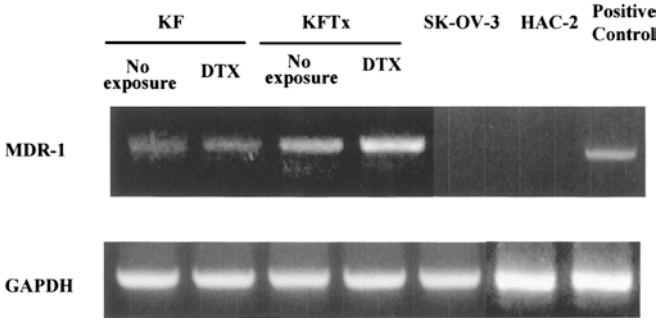

Fig. 1 mRNA expression of MDR-1. KFTx cells showed higher expression of MDR-1 mRNA. After exposure to DTX for 2 h, the expression of MDR-1 increased. The expression was not observed in SK-OV-3 or HAC-2 cells. KOC-7C cells are shown as a positive control

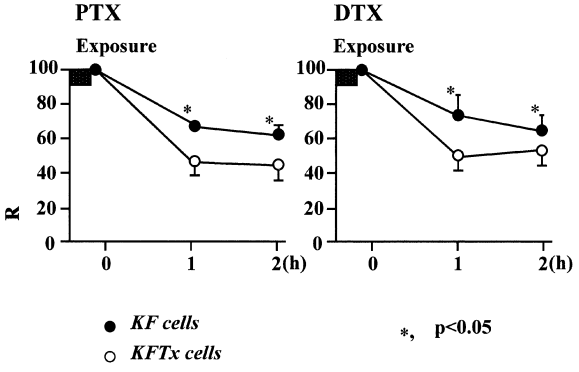

Fig. 2 Intracellular accumulation of PTX and DTX was measured by high-performance liquid chromatography and the residual rate (R) of each drug was calculated. The residual rates (%) of PTX and DTX were significantly lower in KFTx cells than in KF cells over 1 h after exposure to each drug

Table 1 IC_{50} values of PTX and DTX in each cell line

Cell line	PTX (nM)	DTX (n*M*)
KF	128.6	48
KFTx	705.3	351
SK-OV-3	42.0	30
HAC-2	6900.0	2850

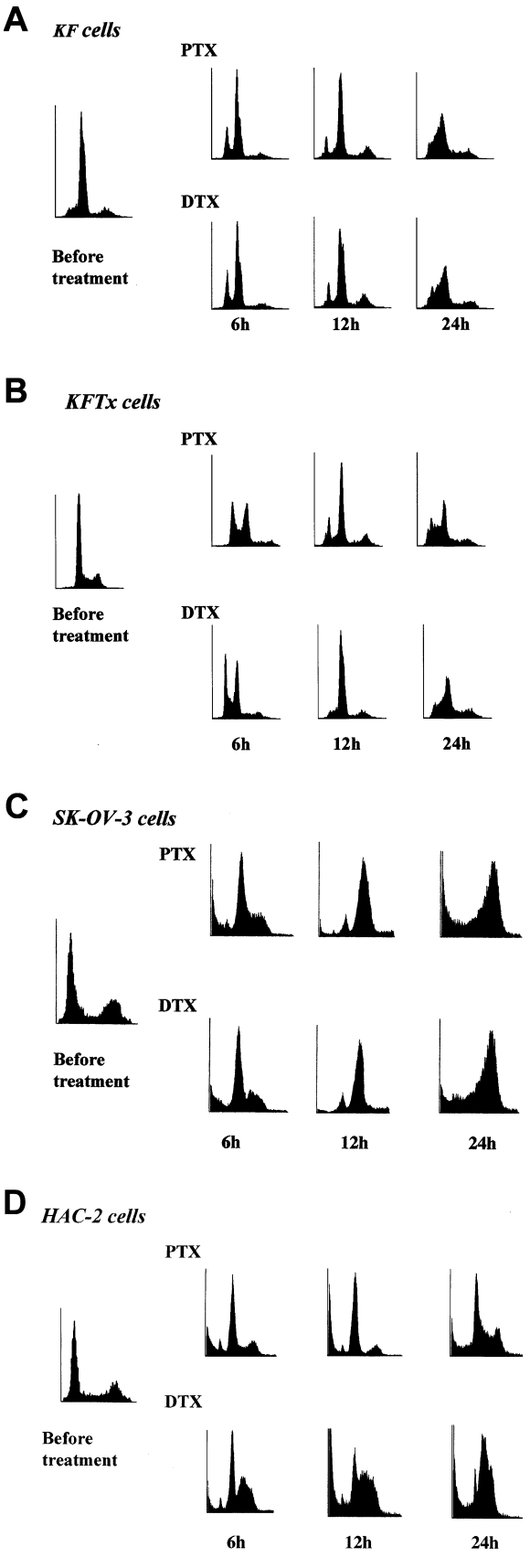

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Fig. 3A–D Flow cytometric analysis indicated no differences in cell cycle distribution following exposure to PTX and to DTX. Accumulation of cells in pre-G_1 6 h after exposure to the drugs and in G_2M 12 h after exposure were observed in KF cells (**A**), KFTx cells (**B**), SK-OV-3 cells (**C**) and HAC-2 cells (**D**) equally

Discussion

Several clinical trials have suggested that DTX is effective in PTX-resistant cancer [2, 17, 25]. The overall response rate was 23% in patients with PTX-resistant Mullerian carcinoma [15]. Incomplete cross-resistance to DTX in PTX-resistant tumors has also been reported

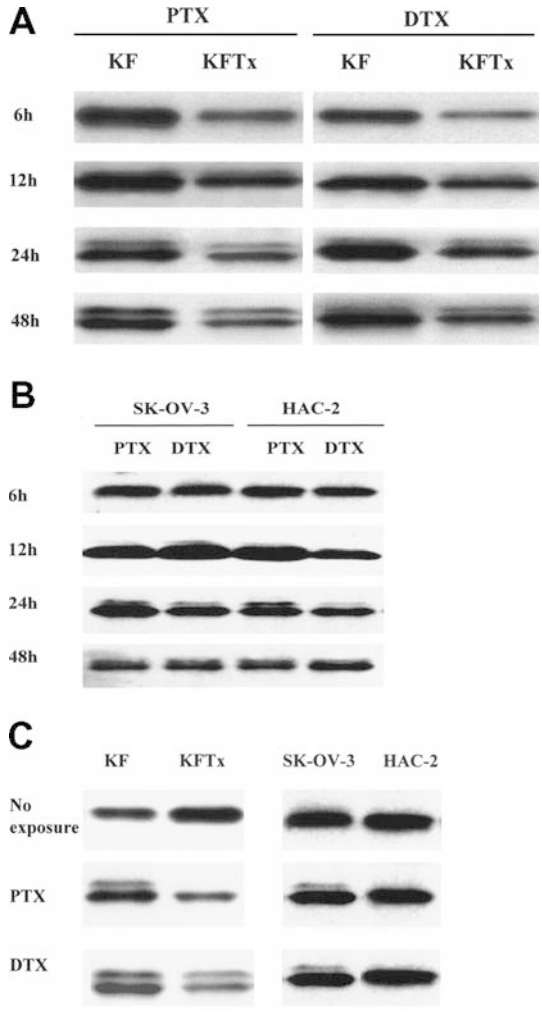

Fig. 4A–C Bcl-2 phosphorylation was detected by Western blotting over 12 h after exposure to the two agents at their IC_{50} values in each cell line (**A**, **B**). Bcl-2 phosphorylation was observed in KFTx cells after exposure for 24 h to 52 n*M* DTX (equivalent to the clinical dose), but was not after exposure to 268 n*M* PTX (equivalent to the clinical dose) (C). In HAC-2 cells, Bcl-2 phosphorylation was not observed after exposure to DTX or PTX at their clinical doses

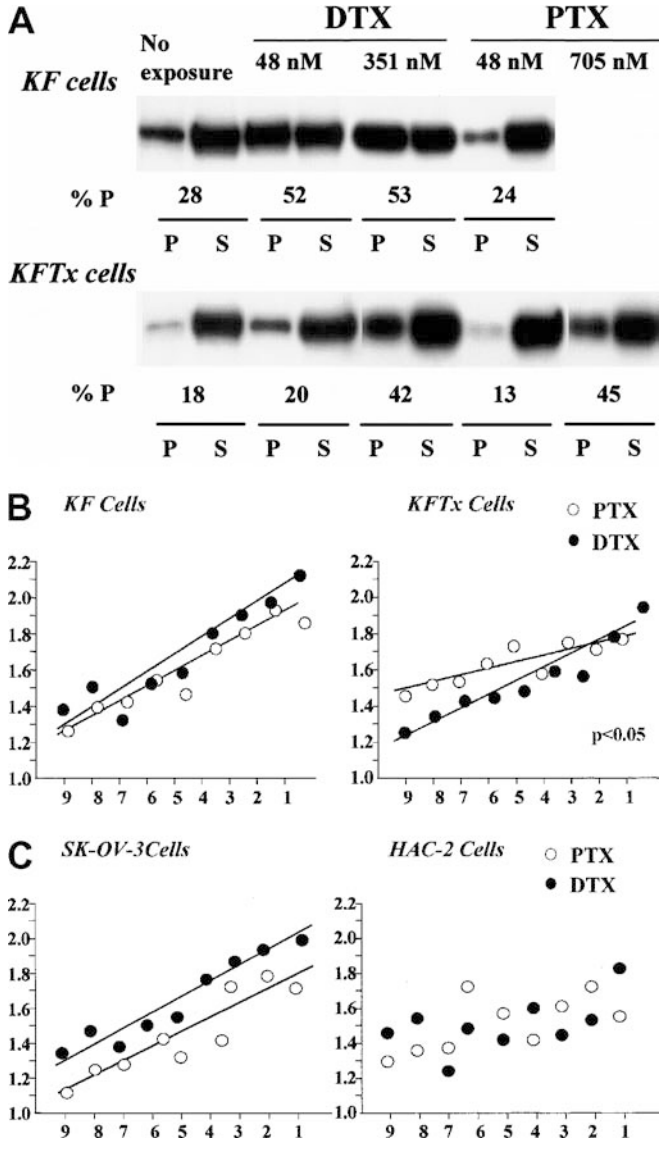

Fig. 5A–C Tubulin polymerization after exposure to PTX or DTX in KF cells. Soluble (*S*) and polymerized (*P*) fractions of β-tubulin were determined by the β-tubulin polymerization assay and the percentage of polymerized to total tubulin (%P) calculated (**A**). The slopes of the regression lines (as indicators of the polymerization activities of PTX and DTX) were compared. The *x*-axis shows the dilution rate on a semi-log scale of (1.5^n). In KF cells, the activities of PTX and DTX did not differ. In KFTx cells, the slope for PTX was significantly less than that in KF cells, but the slope for DTX was not significantly different (**B**). The slope did not differ between PTX and DTX in SK-OV-3 cells. In HAC-2 cells, β-tubulin polymerization was not significantly correlated with the concentration of PTX or DTX, suggesting that tubulin reaction might contribute the resistance in HAC-2 cells (**C**)

[25]. DTX is therefore considered to be an effective agent for the treatment of PTX-resistance cancer, but its mechanism of action remains unknown.

In the present study, the IC_{50} of DTX in PTX-resistant cells was 7.3-fold greater than that in the parental cells, indicating cross-resistance between these agents. MDR-1, which encodes p-glycoproteins, is known to contribute to PTX resistance [6]. We have previously found that the MDR-1 gene is present only in PTX-resistant cells and its expression is increased after exposure to PTX [15, 16]. In the present study, MDR-1 mRNA was also clearly present in KFTx cells and its expression increased after exposure to DTX. In addition, residual cellular accumulation of DTX was significantly lower in KFTx cells than in KF cells. Those findings suggest that DTX efflux via the MDR-1 gene contributes to the resistance in KFTx cells. We used another two cell lines that show no MDR-1 gene expression. HAC-2 cells which showed resistance to PTX and DTX may have another resistance mechanism.

Bcl-2 phosphorylation has been shown to be an important determinant of the efficacy of PTX [3, 22]. Deletion of the Bcl-2 phosphorylation loop domain has also been shown to increase resistance of leukemia cells to PTX-mediated apoptosis [27]. We found that Bcl-2 phosphorylation occurred equally after exposure to PTX or DTX in all cell lines. Flow cytometric analysis showed that alteration in cell cycle distribution did not differ between PTX and DTX exposure. Additionally, the alteration in cell cycle distribution in resistant cell lines was similar to that in sensitive cell lines. Therefore, it is suggested that the cytotoxic mechanism of DTX is similar to that of PTX.

The goal of this study was to determine whether DTX is effective in PTX-resistant cells. Bcl-2 phosphorylation is associated with sensitivity to both PTX and DTX. Interestingly, after exposure to PTX or DTX at clinical doses, Bcl-2 phosphorylation was induced only by DTX in KFTx cells. In contrast, Bcl-2 phosphorylation did not occur after exposure to DTX or PTX at clinical doses in HAC-2 cells. We also examined tubulin polymerization to assess drug activity. In KFTx cells, tubulin polymerization on exposure to PTX significantly decreased, but did not decrease in response to DTX. In HAC-2 cells, β-tubulin polymerization was not correlated with the concentration of PTX or DTX. Altered microtubule dynamics and/or altered binding of PTX has previously been found to constitute a mechanism of resistance [9, 11, 24, 26]. These findings suggest that PTX resistance may be associated not only with the p-glycoprotein-mediated efflux system but also with the tubulin reaction. Therefore, DTX may be effective in PTX-resistant cells with a p-glycoprotein-mediated efflux system. On the other hand, DTX may not be effective in PTX-resistant cells with an altered tubulin reaction. DTX has been shown to have a 1.9-fold greater binding affinity for β-tubulin and to show 2.0-fold greater depolymerization inhibition compared with PTX [26]. Because of a difference in the activity in PTX-resistant cells, DTX is considered to be more potent than PTX.

While the mechanism of cytotoxicity does not differ between PTX and DTX in ovarian cancer cells, DTX may be effective in PTX-resistant cells with an efflux system, because DTX has a greater potential for cytotoxicity.

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