**Regular Article**

Paclitaxel-resistance Conferred by Altered Expression of Efflux and Influx Transporters for Paclitaxel in the Human Hepatoma Cell Line, HepG2

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Summary: Paclitaxel-resistant HepG2 (PR-HepG2) cells were established by long-term exposure of HepG2 cells to paclitaxel and expression and function of efflux (P-glycoprotein, MRP2) and influx (OATP1B3) transporters for paclitaxel were examined to understand the mechanisms underlying the resistance. mRNA expression of P-glycoprotein (P-gp) increased in PR-HepG2 more than in HepG2 cells, while that of MRP2 did not change. Interestingly, mRNA expression of OATP1B3 drastically decreased in PR-HepG2 cells. [3H]Paclitaxel uptake was less in PR-HepG2 than in HepG2 cells and the uptake in both cells increased by metabolic inhibition. The uptake of [3H]paclitaxel and rhodamine 123 increased by verapamil, a P-gp inhibitor. Probenecid, an MRP inhibitor, did not affect [3H]paclitaxel uptake in both cells. Sulfobromophthalein, an OATP1B3 inhibitor, inhibited [3H]paclitaxel uptake in HepG2 but not in PR-HepG2 cells. Cytotoxicity studies showed that the resistance of PR-HepG2 cells to paclitaxel was reversed by verapamil. PR-HepG2 cells showed cross-resistance to doxorubicin, a P-gp substrate, but not to cisplatin. These results indicate that enhanced expression and function of P-gp may be a predominant mechanism of paclitaxel resistance in PR-HepG2 cells and the reduced influx via OATP1B3 may also serve to lower intracellular paclitaxel concentration in cooperation with P-gp-mediated efflux.

Keywords: paclitaxel; multidrug resistance; HepG2 cells; P-glycoprotein (MDR1/ABCB1); multidrug resistance-associated protein 2 (MRP2/ABCC2); organic anion transporting polypeptide 1B3 (OATP1B3/SLC01B3)

**Introduction**

Paclitaxel is an anticancer drug originally derived from the pacific yew tree (*Taxus brevifolia*). It stabilizes microtubules and inhibits depolymerization back to tubulin, resulting in mitotic inhibition. Such an effect causes cell cycle arrest in G2/M phase and finally induces cell death through an apoptotic pathway. Paclitaxel is now widely used as an effective chemotherapeutic agent for the treatment of common cancers such as of the breast, lungs and ovaries. Hepatocellular carcinoma is one of the most prevalent cancers and many patients develop either unresectable or metastatic disease. Therefore, effective chemotherapeutic drugs are greatly desired. However, only a few chemotherapeutic agents show therapeutic effect in the treatment of advanced hepatocellular carcinoma. So far, some clinical studies have been carried out to investigate the efficacy and toxicity of paclitaxel in hepatocellular carcinoma patients. Though the clinical outcomes differed depending on the study design including dose and schedule, the efficacy of paclitaxel on hepatocellular carcinoma has not been established. Recently, the effects of various new formulations containing paclitaxel such as liposomes, lipid nanocapsules and block copolymer micelles on hepatocellular carcinoma have been extensively studied,
in addition to paclitaxel itself.6–10

Resistance of tumor cells to anticancer drugs is a serious problem in cancer chemotherapy and resistance to taxane is considered to be mediated by various mechanisms such as altered intracellular drug levels, variation in tubulin structure, altered signal transduction and apoptotic pathways.11–13 Though there are a few studies on the cytotoxic effects of paclitaxel on hepatoma cell lines including HepG2,14,15 information concerning the mechanisms underlying paclitaxel resistance in hepatoma cells is still lacking.

The present study first established paclitaxel-resistant HepG2 (PR-HepG2) cells, by long-term treatment of HepG2 cells with paclitaxel. HepG2 is a cell line derived from a well differentiated human hepatoblastoma and retains many normal biochemical functions of human hepatocytes.16,17 Generally, the cytotoxicity of an anticancer drug is related to its intracellular concentration. Therefore, after establishing PR-HepG2 cells, we examined paclitaxel transport in the cells. Among various transporters expressed in hepatocytes, an efflux transporter P-glycoprotein (P-gp; MDR1/ABCB1) is well known to transport paclitaxel as a substrate.13,18,19 Increased expression of P-gp is an important, though not the sole, mechanism for paclitaxel resistance in various cancer cells as well as in clinics.13,20 Another possible transporter involved in paclitaxel efflux from the hepatocytes is multidrug resistance-associated protein 2 (MRP2/ABCC2) and MRP2 may be related to paclitaxel resistance.21,22 In contrast to the role of efflux transporters in the cellular handling of paclitaxel, the mechanism by which paclitaxel enters hepatocytes has received relatively little attention because of its hydrophobic nature. Recently, Smith et al.23 found that the organic anion transporting polypeptide 1B3 (OATP1B3/SLCO1B3), but not OATP1B1 may be important to the uptake of paclitaxel into hepatocytes. Therefore, in the present study the expression and function of efflux (P-gp, MRP2) and influx (OATP1B3) transporters for paclitaxel were examined and compared in HepG2 and PR-HepG2 cells to understand the mechanisms underlying paclitaxel resistance.

Materials and Methods

Materials: Dulbecco’s Modified Eagle’s Medium (DMEM), trypsin-EDTA and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Paclitaxel was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sulfobromophthalein (BSP), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, inner salt (XTT), N-methyl dibenzopyrazine methyl sulfate (PMS), cisplatin, doxorubicin hydrochloride and probenecid were from Sigma-Aldrich (St. Louis, MO, USA). Verapamil was purchased from Nacalai Tesque (Kyoto, Japan). Rhodamine 123 and 2-deoxy-D-glucose were from Kanto Chemical (Tokyo, Japan) and sodium azide, from Katayama Chemical (Tokyo, Japan). A Nucleic acid purification kit (Mag Extractor-RNA), RT-PCR kit (Rever Tra Dash) and SYBR<sup>®</sup> Green Realtime PCR Master Mix were purchased from TOYOBO (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

HepG2 cell culture: HepG2 cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and cultured in DMEM containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin (DMEM medium), in an atmosphere of 5% CO<sub>2</sub>–95% air at 37°C and subcultured every 6–7 days using 0.25% trypsin and 1 mM EDTA. Fresh medium was replaced every 2 or 3 days and the cells were used for the experiments on the fifth to eighth day after seeding.

Establishment of paclitaxel-resistant HepG2 (PR-HepG2) cells: HepG2 cells were seeded at a density of 3000 cells/100 mm dish and cultured in DMEM medium containing 3 nM paclitaxel for two weeks. After two weeks, all the cells were subcultured in a 35 mm dish and cultured for two more weeks. 100 × 10<sup>4</sup> cells were then subcultured into a 100 mm dish and the cells were subcultured every 7–8 days at a density of 100 × 10<sup>4</sup> cells/100 mm dish, using DMEM medium containing 3 nM paclitaxel.

XTT-based cytotoxicity assay: Cytotoxicity of the anticancer drugs was determined by the XTT tetrazolium/formazan assay as described previously.24 This method is based on cleavage of XTT by metabolically active cells. Briefly, HepG2 and PR-HepG2 cells were seeded at a density of 5 × 10<sup>4</sup>/well in a 24-well flat-bottomed plate and incubated for 24 hrs. The medium was replaced with serum-free DMEM medium containing paclitaxel (0–300 nM), cisplatin (0–300 μM) or doxorubicin (0–30 μM) and the cells were further incubated at 37°C for 48 hrs. Following incubation, the medium was removed and the cells were washed twice with phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>·1·5mMK H<sub>2</sub>PO<sub>4</sub>, 1·5 mM KH<sub>2</sub>PO<sub>4</sub>, 0·1 mM CaCl<sub>2</sub>, and 0·5 mM MgCl<sub>2</sub>·pH 7·4; PBS buffer) supplemented with 5 mM D-glucose (PBS-G buffer) and then 0·2 mL 250 μM XTT in PBS-G buffer containing 10 μM PMS was added to each well. The cells were incubated for 30 min at 37°C. After transfer of the reaction solution to a microplate well, the amount of orange formazan dye produced was quantitated using a VERSA max™ microplate reader (Molecular Devices, Toronto, Canada) to measure absorbance at a wavelength of 490 nm. Reference absorbance (nonspecific reading) was measured at a wavelength of 650 nm. The percentage of viability was calculated as follows: Viability (%) = (A<sub>490</sub> − A<sub>650</sub><sub>untreated</sub>)/(A<sub>490</sub> − A<sub>650</sub><sub>control</sub>) × 100. The half maximal inhibitory concentration (IC<sub>50</sub>) of each anticancer drug is related to its intracellular concentration.
cer drug was determined by the Hill equation, \( V = 100/\left(1 + ([I]/IC_{50})^n\right) \), where \([I]\) is the concentration of each anticancer drug, \( V \), viability in the presence of each anticancer drug and \( n \), the Hill coefficient. The KaleidaGraph™ program (Version 3.08, Synergy Software, PA, USA) was used for curve-fitting. IC50 was assessed from the curve-fitting to the above-mentioned equation.

### Analysis of mRNA expression of albumin and transporters in HepG2 cells:

mRNA expression of albumin, MDR1, MRP2 and OATP1B3 was determined as described previously.25,26 Total RNA was extracted from HepG2 cells with Mag Extractor-RNA. Total RNA (0.2 μg) was used for RT to generate cDNA using Rever Tra Dash and the generated RT cDNA was used for PCR amplification with a Program Temp control system PC-707 (ASTEC, Fukuoka, Japan). The primers for albumin were sense, 5′-GCTCAGATTTCCAGTGGTCC -3′, and antisense, 5′-GTGGACATTTGTAAGATGTGTG-CACT-3′ (expected size of PCR product, 645 bp). The conditions for PCR were denaturation, 95°C for 30 sec; annealing, 60°C for 30 sec and extension, 72°C for 30 sec (20 cycles). The primers for MDR1 (P-gp) were sense, 5′-GTCACTTGGAGAAAGAAATCATG-3′ and antisense, 5′-ATTCCAAGGCTAGAAACATA-GT-3′ (expected size of PCR product, 479 bp). The conditions for PCR were denaturation, 94°C for 35 sec; annealing, 60°C for 35 sec and extension, 72°C for 35 sec (33 cycles). The primers for MRP2 were sense, 5′-ACACCAACGAGAATGTGTC-3′ and antisense, 5′-CCAGGCTTCCAATCTC-3′ (expected size of PCR product, 659 bp). The conditions for PCR were denaturation, 94°C for 1 min; annealing, 62°C for 1 min and extension, 72°C for 1 min (24 cycles). The primers for OATP1B3 were sense, 5′-TCATAAACCTTGTGTC-3′ and antisense, 5′-TGTTGAGGGCATTGTCTTG-3′ (expected size of PCR product, 481 bp). The conditions for PCR were denaturation, 94°C for 1 min; annealing, 67°C for 1 min and extension, 72°C for 1 min (31 cycles). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were sense, 5′-GCAGGGGAGGCCCCAAGGG-3′ and antisense, 5′-TGCCAGCCCCAGCTGAAAG-3′ (expected size of PCR product, 567 bp). The conditions for PCR were denaturation, 94°C for 1 min; annealing, 69°C for 1 min and extension, 72°C for 1 min (20 cycles). PCR products with or without reverse transcription were separated by electrophoresis through 2.0% agarose gels and visualized under ultraviolet light with ethidium bromide.

### Evaluation of mRNA expression by real-time PCR:

Total RNA was extracted from HepG2 and PR-HepG2 cells cultured with 3 nM paclitaxel for 78 and 113 days by Mag Extractor-RNA as described previously.27 Total RNA (0.1 μg) in a final volume of 10 μL was reverse transcribed into cDNA using Rever Tra Ace. Real-time PCR was performed on a BioFlux LineGene system using SYBR Green. Reaction mixtures consisted of 2 μL cDNA, 10 μL SYBR Green and primers in a final volume of 20 μL. PCR conditions except for OATP1B3 were: initial denaturation in one cycle of 1 min at 95°C followed by 40 cycles of 5 sec at 95°C (denaturation), 5 sec at 60°C (annealing), and 15 sec at 72°C (extension). PCR conditions for OATP1B3 were: initial denaturation in one cycle of 1 min at 95°C followed by 45 cycles of 5 sec at 95°C (denaturation), 5 sec at 63°C (annealing), and 15 sec at 72°C (extension). After the cycles, a melting curve was checked to confirm the single product. The primers were: sense, 5′-CCCATCATGCAATAGCAGG -3′ and antisense, 5′-TGTTCAGCTTTGCTCCTGA-3′ for MDR1 (P-gp), sense, 5′-TGACAGTTTAGAAAGG.azure-CACAT-3′ and antisense, 5′-AGCTCTTCCTCGCGCT- CTCT-3′ for MRP2; sense, 5′-GTCAGTCATGGG- CTTTGCA-3′ and antisense, 5′-ACACCAACGAGGT-CCTTAGG-3′ for OATP1B3; sense, 5′-CCACCAATGGAATCC-3′ and antisense, 5′-TGGGATTCTCCA-TTGATGACAA-3′ for GAPDH. The expression level of each mRNA was normalized by that of GAPDH, a housekeeping gene.

### Uptake of [1H]paclitaxel in HepG2 and PR-HepG2 cells:

Uptake experiments were performed as described previously,25 using cells grown on 24-well plates. After removal of the culture medium, each well was washed twice with PBS-G buffer and preincubated in the absence or presence of the inhibitor for 15 min at 37°C. Preincubation conditions in the presence of the inhibitor were as follows: 10 mM sodium azide and 5 mM 2-deoxy-D-glucose in PBS buffer, 100 μM verapamil and 1 mM probenecid in PBS-G buffer containing 0.5% DMSO, 10 μM BSP in PBS-G buffer. The same vehicles were used for each control experiment. After preincubation, [1H]paclitaxel with or without the inhibitor was added, and the cells were incubated at 37°C for a specified period. After incubation, the uptake buffer was aspirated and the cells were rinsed rapidly three times with ice-cold PBS buffer. The cells were scraped with a rubber policeman into 0.3 mL 0.1 M NaOH and the wells were rinsed again to improve the recovery of the cells. For measurement of [1H]paclitaxel, 3 mL ACS II (Amersham Pharmacia Biotech, Buckinghamshire, UK) were added and radioactivity was measured by liquid scintillation counting. Protein concentration was measured by the method of Bradford with bovine serum albumin as the standard.28

### Uptake of rhodamine 123 by HepG2 and PR-HepG2 cells:

Uptake experiments were performed as described previously,25 using cells grown on a 35 mm dish. After removal of the culture medium, each dish was washed and preincubated with PBS-G buffer in the absence or presence of P-gp inhibitor, 100 μM verapamil (0.5% DMSO) at 37°C for 15 min. After preincubation, PBS-G buffer containing rhodamine 123 (10 μM) was ad-
ded to each dish and the cells were incubated at 37°C for a specified period. At the end of incubation, the uptake buffer was aspirated and the dishes were rinsed rapidly two times with ice-cold PBS buffer. 0.1% Triton X-100 in PBS buffer without CaCl₂ and MgCl₂ (PBS(−) buffer) was added to each dish and the cells were scraped with a rubber policeman. The dishes were rinsed again to improve cell recovery. The cells were solubilized in 0.1% Triton X-100 in PBS(−) buffer for 30 min at room temperature and centrifuged at 5,600 g for 5 min. The supernatant was used for fluorescence and protein assays. The fluorescence of rhodamine 123 was measured using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at excitation and emission wavelengths of 485 and 546 nm, respectively. Protein content was determined by the Lowry method.²⁹

**Statistical analysis:** Data are expressed as means ± SE. Statistical analysis was performed by Student’s t-test or one-way ANOVA followed by Tukey’s test for multiple comparison. The level of significance was set at *p < 0.05 or **p < 0.01.

**Results**

To establish PR-HepG2 cells, HepG2 cells were cultured in the presence of 3 nM paclitaxel. Figure 1 shows the sensitivity of wild-type (untreated) and paclitaxel-treated HepG2 cells to paclitaxel and calculated IC₅₀ are summarized in Table 1. According to the exposure period, HepG2 cells became resistant to paclitaxel and when the cells were continuously exposed to 3 nM paclitaxel for more than 100 days, IC₅₀ increased to about 10- to 15-fold that observed for untreated HepG2 cells.

**Figure 2** shows the expression of mRNAs of albumin and transporters in HepG2 and PR-HepG2 cells. In HepG2 cells, mRNA expression of albumin, MDR1 (P-gp), MRP2 and OATP1B3 was observed (Fig. 2A). In Figure 2B, mRNA expression of each transporter normalized by GAPDH is shown. In PR-HepG2 cells exposed to paclitaxel for 78 or 113 days, the expression of MDR1 mRNA was found to significantly increase compared to untreated HepG2 cells. The expression of MRP2 did not change by paclitaxel exposure. Interestingly, the expression of OATP1B3 was drastically reduced in PR-HepG2 cells. At 37 day after exposure, OATP1B3 mRNA expression was about half that in untreated HepG2 cells (data not shown), indicating the decrease in OATP1B3 mRNA expression to possibly depend on the time of exposure. In subsequent studies, PR-HepG2 cells exposed to 3 nM paclitaxel for more than 100 days were used.

Using HepG2 and PR-HepG2 cells, we examined the uptake of [³H]paclitaxel in these cells. **Figure 3** shows the time course of [³H]paclitaxel uptake and the uptake was significantly lower in PR-HepG2 than HepG2 cells (Fig. 3). The uptake of [³H]paclitaxel increased by treatment with sodium azide and 2-deoxy-D-glucose (metabolic inhibition) to essentially the same extent in both HepG2 and PR-HepG2 cells (Fig. 4).

To clarify the roles of hepatic transporters in cellular accumulation of paclitaxel, the effects of various transport inhibitors were examined. First, the effects of verapamil on [³H]paclitaxel uptake were examined. The uptake of [³H]paclitaxel increased by verapamil in both cells, indicating the involvement of P-gp in paclitaxel efflux from the cells (Fig. 5A). The effects of verapamil on the uptake of rhodamine 123, a typical P-gp substrate were thus examined. Similar to the uptake of paclitaxel, rhodamine 123 uptake was less in PR-HepG2 than HepG2 cells and uptake increased by verapamil (Fig. 5B). **Figure 6** shows the effects of probenecid on [³H] paclitaxel uptake in HepG2 and PR-HepG2 cells. However, no significant effect of probenecid could be seen in either cell. **Figure 7** shows the effect of BSP on
Fig. 2. The expression of mRNAs of albumin and transporters in HepG2 cells (A) and effects of paclitaxel treatment on the expression of mRNAs of MDR1 (a), MRP2 (b) and OATP1B3 (c) in HepG2 cells (B).

(A) Total RNA was isolated and RT-PCR analysis was performed to evaluate the expression of each mRNA in HepG2 cells. Lane 1, molecular marker; lanes 2–4, RNA samples extracted from HepG2 cells. (B) HepG2 cells were cultured in the absence (open column) or presence of 3 nM paclitaxel for 78 and 113 days (closed column). The bar graph shows the relative ratio (treated/untreated) of each mRNA expression estimated by real-time PCR method. Ratio was calculated after normalization by GAPDH, a housekeeping gene. Each value represents the mean ± SE of 3 RNA samples. *p < 0.05, **p < 0.01, significantly different from the untreated.

Fig. 3. Time course of [3H]paclitaxel uptake in HepG2 and PR-HepG2 cells
HepG2 (open circle) and PR-HepG2 (open triangle) cells were incubated with 6 nM [3H]paclitaxel. Each value represents the mean ± SE of 3 monolayers. *p < 0.05, **p < 0.01, significantly different from HepG2 cells at each time.

Fig. 4. Effect of sodium azide and 2-deoxy-D-glucose (metabolic inhibition) on the uptake of [3H]paclitaxel in HepG2 and PR-HepG2 cells
HepG2 (circle) and PR-HepG2 (triangle) cells were incubated with 3 nM [3H]paclitaxel in the absence (open symbol; control) or presence of 10 mM sodium azide and 5 mM 2-deoxy-D-glucose (closed symbol). Each value represents the mean ± SE of 3 monolayers. **p < 0.01 and ††p < 0.01, significantly different from the control in HepG2 and PR-HepG2 cells at each time, respectively.

[3H]paclitaxel uptake. The uptake of [3H]paclitaxel in PR-HepG2 cells, in which the expression of OATP1B3 had almost disappeared, was not affected by BSP. [3H] paclitaxel uptake in HepG2 cells was significantly inhibited by BSP. We also examined the effects of BSP on the initial uptake (1, 2 and 5 min) of [3H]paclitaxel in HepG2 and PR-HepG2 cells. The uptake of [3H]paclitaxel in
Mechanism of Paclitaxel-resistance in HepG2 Cells

HepG2 cells was almost linear up to 5 min and the uptake for 5 min was significantly inhibited by BSP to 44.9 ± 7.2% of control (p < 0.01, mean ± SE of 3 monolayers). The initial uptake of [3H]paclitaxel in PR-HepG2 cells was not affected by BSP.

Because the cellular uptake of paclitaxel increased by verapamil, we examined the effects of verapamil on the sensitivity of HepG2 and PR-HepG2 cells to paclitaxel. In the presence of verapamil, the paclitaxel concentration-cell viability curve shifted to the left, especially in PR-HepG2 cells, indicating paclitaxel resistance of PR-HepG2 cells to be reversed by verapamil (Fig. 8). Calculated IC_{50} are summarized in Table 2.

The cross-resistance of PR-HepG2 cells to other anticancer drugs was examined. There was no difference in sensitivity to cisplatin for HepG2 and PR-HepG2 cells (Fig. 9A). PR-HepG2 cells showed resistance to doxorubicin, a substrate of P-gp (Fig. 9B). Calculated IC_{50} for each of the anticancer drugs are summarized in Table 3.

Discussion

In this study, the mechanisms underlying the resistance to paclitaxel were examined in HepG2 cells, mainly from the viewpoint of transporters for paclitaxel. For this purpose, we first established PR-HepG2 cells by long-term exposure of wild-type HepG2 cells to 3 nM paclitaxel. In accordance with the culture period, HepG2 cells became more resistant to paclitaxel and after 3–5
Effects of verapamil on the sensitivity of HepG2 and PR-HepG2 cells to paclitaxel

HepG2 (circle) and PR-HepG2 (triangle) cells were incubated with various concentrations of paclitaxel in the absence (open symbol; control) or presence of 1 μM verapamil (closed symbol) for 48 hrs. Cell viability was estimated by XTT assay after 48 hr treatment. Each value represents the mean ± SE of 3 monolayers.

Table 2. Effects of verapamil (VRP) on paclitaxel-induced cytotoxicity in HepG2 and PR-HepG2 cells

<table>
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<tr>
<th>Cells (treatment)</th>
<th>IC_{50} (nM)</th>
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<tbody>
<tr>
<td>HepG2 (without VRP)</td>
<td>24.7 ± 3.3</td>
</tr>
<tr>
<td>HepG2 (with VRP)</td>
<td>18.5 ± 2.8</td>
</tr>
<tr>
<td>PR-HepG2 (without VRP)</td>
<td>249.1 ± 27.5</td>
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| PR-HepG2 (with VRP)          | 52.5 ± 5.8   **

IC_{50} (concentrations of paclitaxel causing decrease in cell viability to 50% of control) were calculated by fitting the data in Figure 8 to Hill’s equation. Each value represents the mean ± SE of 3 monolayers. **p < 0.01, significantly different from the IC_{50} value in PR-HepG2 (without VRP).

IC_{50} for paclitaxel became 10- to 15-fold higher than in HepG2 cells (Fig. 1 and Table 1). In preliminary experiments, we examined other concentrations of paclitaxel, 1 nM and 5 nM, to establish PR-HepG2 cells. However, when HepG2 cells were treated with 1 nM paclitaxel, cell growth rate and apparent morphology did not change from those noted for control cells. When cells were treated with 5 nM paclitaxel, the growth rate was reduced markedly and finally all the cells died (data not shown). Therefore, the concentration of paclitaxel, in this case 3 nM, would be a critical factor to establish PR-HepG2 cells.

To carry out the experiments over a long period, the stability of paclitaxel resistance in PR-HepG2 cells is important practically. For example, Garcia-Martin et al. studied paclitaxel resistance in human colorectal cancer cells Caco-2, and suggested that acquired resistance to paclitaxel was associated with induction of cytochrome P450 2C8 (CYP2C8), a paclitaxel metabolizing enzyme. They examined the reversibility of the resistance and showed that, when paclitaxel was removed from the culture medium, mRNA expression of CYP2C8, but not that of MDR1, dropped sharply (within 2 hours) and resistance to paclitaxel decreased. Therefore, we tested

Table 3. Cross-resistance to cisplatin and doxorubicin in HepG2 and PR-HepG2 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC_{50} (μM)</th>
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<tr>
<td></td>
<td>Cisplatin</td>
</tr>
<tr>
<td>HepG2</td>
<td>37.1 ± 4.6</td>
</tr>
<tr>
<td>PR-HepG2</td>
<td>44.4 ± 5.1</td>
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</table>

IC_{50} were calculated by fitting the data in Figure 9 to Hill’s equation. Each value represents the mean ± SE of 4 monolayers. *p < 0.05, significantly different from the IC_{50} value in HepG2 cells.

Fig. 8. Effects of verapamil on the sensitivity of HepG2 and PR-HepG2 cells to paclitaxel

Fig. 9. Comparison of the sensitivity of HepG2 and PR-HepG2 cells to cisplatin (A) and doxorubicin (B)

HepG2 (circle) and PR-HepG2 (triangle) cells were incubated in various concentrations of cisplatin (A) and doxorubicin (B) for 48 hrs. Cell viability was estimated by XTT assay after 48 hr treatment. Each value represents the mean ± SE of 4 monolayers.
the reversibility of paclitaxel resistance in PR-HepG2 cells. However, when PR-HepG2 cells were cultured in the medium without paclitaxel for 1 or 4 weeks, resistance to paclitaxel as well as the expression of mRNAs of P-gp, MRP2 and OATP1B3 did not change under our experimental conditions (data not shown). When PR-HepG2 cells were stored in liquid nitrogen and used after thawing, resistance to paclitaxel did not change. Thus, paclitaxel resistance of PR-HepG2 cells may be phenotypically stable. These findings may indicate that paclitaxel resistance of PR-HepG2 cells is due to the selection of resistant cells from wild-type, heterogeneous HepG2 cell populations, in contrast to the above mentioned acquired resistant cells from wild-type, heterogeneous HepG2 cell lines.30 We also examined the effects of short-term paclitaxel treatment (10 nM, 1 μM, or 10 μM for 24 hours) on MDR1 mRNA expression in wild-type HepG2 cells. However, no change in mRNA expression was observed (data not shown), indicating that induction of MDR1 mRNA may not occur under the experimental conditions used. Though further studies are needed, these results support the selection mechanism.

HepG2 retains many functional characteristics of human hepatocytes such as albumin production.16 In this study, mRNA expression of albumin as well as that of hepatic transporters were confirmed by RT-PCR (Fig. 2A). In PR-HepG2 cells, the enhanced and reduced expression of mRNA for P-gp and OATP1B3, respectively, was observed, while that of MRP2 did not change (Fig. 2B). Reduction in OATP1B3 mRNA expression was particularly remarkable. The overexpression of P-gp is often observed and is the cause, though not necessarily, of resistance to antimitotic agents.5) We thus examined whether these transporters are involved in paclitaxel handling in HepG2 and PR-HepG2 cells and/or related to paclitaxel resistance.

We first examined the uptake of [3H]paclitaxel in HepG2 and PR-HepG2 cells and found that the uptake in PR-HepG2 was about the half that in HepG2 cells (Fig. 5B). Though the expression of P-gp protein should also be examined, these results indicate the enhanced expression and function of P-gp in PR-HepG2 cells to possibly be related to paclitaxel resistance of the cells. This notion was supported by the cytotoxicity studies of paclitaxel, showing that the resistance of PR-HepG2 cells to paclitaxel was potently reversed by the presence of a P-gp inhibitor, verapamil (Fig. 8, Table 2). PR-HepG2 cells showed cross-resistance to doxorubicin, but not to cisplatin (Fig. 9A, B, Table 3). Doxorubicin, an anthracycline antibiotic, is an anticancer drug used for the treatment of various malignancies such as carcinoma of the breast, lungs and ovaries.31,32 Doxorubicin is a substrate of P-gp and MRP,18,33 and doxorubicin resistance was observed in cancer cells overexpressing P-gp and/or MRP.31,34 In this study, the expression of MRP2 was not different for HepG2 and PR-HepG2 cells, indicating that the cross-resistance of PR-HepG2 cells to doxorubicin may be due to enhanced expression of P-gp. PR-HepG2 cells did not show cross-resistance to cisplatin, a substrate of MRP2 but not P-gp.35 Taken together, these results suggest the resistance of PR-HepG2 cells to paclitaxel as well as to doxorubicin to possibly be due mainly to the enhanced expression and function of P-gp.

Paclitaxel is suggested to be a substrate of another efflux transporter MRP2 and MRP2 may be related to paclitaxel resistance.21,22 However, [3H]paclitaxel uptake was not affected by probenecid, a potent and non-specific inhibitor of MRPs, either HepG2 or PR-HepG2 cells (Fig. 6). The expression of MRP2 in PR-HepG2 cells did not differ significantly from that in HepG2 cells (Fig. 2B), indicating that the contribution of MRP2 in paclitaxel efflux and resistance would be, if any, very small in these cells.

OATP1B3 is a transporter expressed on sinusoidal membranes of hepatocytes. Smith et al.23 first reported that OATP1B3 may play an important role for the uptake of paclitaxel into hepatocytes. However, the role of OATP1B3 in paclitaxel transport is still controversial.35,36 Therefore, the effects of BSP, a substrate and an inhibitor of OATP1B3,37,38 on [3H]paclitaxel uptake were examined. In HepG2 cells in which OATP1B3 mRNA expression was observed, [3H]paclitaxel uptake was inhibited by BSP. In contrast, in PR-HepG2 cells in which OATP1B3 mRNA expression decreased nearly to the negligible level, BSP did not affect [3H]paclitaxel uptake (Fig. 7). Thus, OATP1B3 may be involved in paclitaxel uptake in HepG2 cells, and the reduction of OATP1B3 expression may be, in addition to enhanced expression of P-gp, partly related to paclitaxel resistance in PR-HepG2 cells.

Using a human hepatocellular carcinoma cell line showing resistance to paclitaxel (QGY-TR50 cell line), Zhou et al.39 investigated the mechanism of paclitaxel resistance in the cells. They concluded that P-gp could be
a key factor involved in enhanced paclitaxel resistance in QGY-TR50 cells. Takara et al.\(^{40}\) established a paclitaxel-resistant Hela subline by long-term exposure of Hela cells to paclitaxel and suggested that enhanced activity of P-gp may be related to paclitaxel resistance in the cells. Though the cells used were different, our results are compatible with the above mentioned findings and overexpression of P-gp may be an important determinant for conferring paclitaxel resistance in various cancer cells. Garcia-Martin et al.\(^{30}\) examined the mechanism of acquired resistance to paclitaxel in Caco-2 cells and suggested that the overexpression of a paclitaxel metabolizing enzyme CYP2C8 may be more directly related to the resistance than P-gp. Other mechanisms not examined in this study may be involved, including acquisition of mutations in tubulin that attenuates paclitaxel binding, increased expression of specific tubulin isoforms and alterations in signaling pathways associated with microtubule function.\(^{11}\) Further studies are needed to clarify the detailed molecular mechanisms of paclitaxel resistance in PR-HepG2 cells.

In conclusion, paclitaxel-resistant HepG2 cells were established and mechanisms underlying the resistance were examined by focusing on transporters for paclitaxel. Our results suggest that paclitaxel resistance is mainly conferred by increased expression of an efflux transporter P-gp, and partly by reduced expression of an influx transporter OATP1B3. Such a cooperative alteration in efflux and influx transporters in paclitaxel-resistant cancer cells appears a novel and interesting finding, and thus important to understand the molecular mechanisms of drug resistance of cancer cells.

References

22) Lagas, J. S., Vlaming, M. L., van Tellingen, O., Wagenaar, E., Jansen, R. S., Rosing, H., Beijnen, J. H. and Schinkel, A. H.: Multidrug resistance protein 2 is an important determinant of
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