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Research Paper

Organic anion transporting polypeptide 2-mediated uptake of paclitaxel and 2′-ethylcarbonate-linked paclitaxel in freshly isolated rat hepatocytes

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Abstract

Objectives The P-glycoprotein (P-gp) efflux pump plays an important role in paclitaxel detoxification. However, hepatic uptake of paclitaxel mediated by a solute-linked carrier transporter family is still poorly understood in animals and humans. Freshly isolated hepatocyte suspensions are a well established in-vitro model for studying drug transport and xenobiotic metabolism. Therefore, the hepatic uptake of paclitaxel and its P-gp-insensitive prodrug, 2′-ethylcarbonate-linked paclitaxel (TAX-2′-Et), has been characterized using freshly isolated and pregnenolone-16-α-carbonitrile (PCN)-treated hepatocytes in rats.

Methods Paclitaxel and TAX-2′-Et were incubated with rat hepatocyte suspensions in the presence or absence of inhibitors.

Key findings Paclitaxel and TAX-2′-Et showed concentration-dependent uptake in rat hepatocytes. The intrinsic transport capacity was two-fold higher for paclitaxel uptake than for TAX-2′-Et uptake. Rifampicin (a potent inhibitor of organic anion transporting polypeptide (Oatp) 2), but not indometacin (a representative inhibitor of organic anion transporter (Oat) 2 and Oatp1) treatment, significantly inhibited the uptake of paclitaxel and TAX-2′-Et. We characterized the rifampicin-sensitive uptake of paclitaxel and TAX-2′-Et using rat hepatocytes treated with PCN, which dramatically enhances hepatic Oatp2 protein levels. PCN-treated hepatocytes displayed a 1.6-fold greater uptake of paclitaxel and TAX-2′-Et than the vehicle-treated hepatocytes. The uptake of the two compounds was significantly reduced by rifampicin but not by indometacin treatment. These findings demonstrated that the rat Oatp2, but not Oatp1 or Oat2, was a candidate transporter for the hepatic uptake of paclitaxel and TAX-2′-Et.

Conclusions The findings have provided an important step towards identifying a key transporter in hepatic detoxification of paclitaxel and TAX-2′-Et in small animals.

Keywords rat hepatocytes; paclitaxel; organic anion transporting polypeptide 2; P-glycoprotein-insensitive prodrug

Introduction

Paclitaxel (Taxol) has been used for the treatment of a variety of tumours, including those of the breast, ovary and lung.[1] However, multidrug resistance proteins cause tumour resistance against chemotherapy because of higher toxicity-limiting doses of paclitaxel. The poor aqueous solubility of paclitaxel is also a problem for intravenous administration. To overcome these drawbacks, a number of investigators have developed paclitaxel prodrugs and conjugates based on the chemical modification of the hydroxyl groups at position 7 of the baccatin core and/or position 2′ of the paclitaxel side chain.[2–4] In our previous studies, 2′-ethylcarbonate-linked paclitaxel (TAX-2′-Et; Figure 1) showed a low sensitivity to hydrolytic enzymes in human serum, and circumvented P-glycoprotein (P-gp)-mediated efflux of paclitaxel in paclitaxel-resistant cells, demonstrating one of the most important factors of gene-directed enzyme prodrug therapy (GDEPT) strategies that allow the tumour cell-specific cytotoxicity of paclitaxel.[5,6]

Drug detoxification in the liver is limited by hepatic uptake across the sinusoidal membrane, which is followed by metabolism and biliary excretion, so that the hepatic

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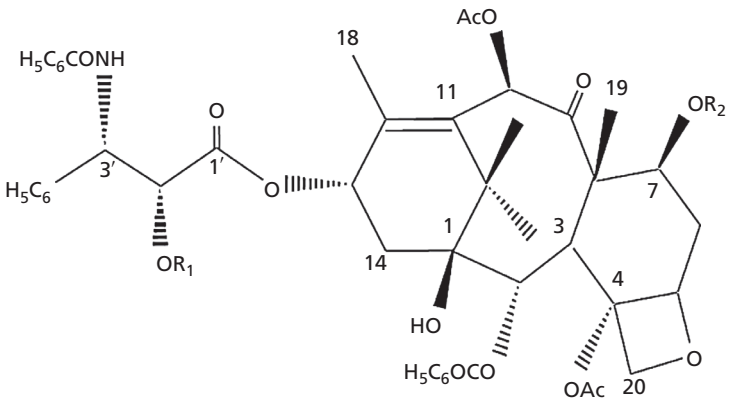

	MW	R_1	R_2
Paclitaxel	854	H	H
2′-Ethylcarbonate-linked paclitaxel	924	$CO_2C_2H_5$	H

Figure 1 Chemical structures of paclitaxel and 2′-ethylcarbonate-linked paclitaxel

uptake rate can be one of the determinants of drug disposition. Transporter-expressing systems, membrane vesicles, and hepatocytes are useful for studying carrier-mediated transport of xenobiotics. Human organic anion-transporting polypeptide (OATP) 8 and human organic anion transporter (OAT) 2 are abundantly expressed in the liver, and are considered to be key molecules in hepatic handling of organic anions. Paclitaxel is a hydrophobic compound without an anionic moiety at physiological pH, however, OATP8-expressing *Xenopus laevis* oocytes accumulate 3.3-fold more paclitaxel than water-injected oocytes. A paclitaxel derivative, docetaxel, is also reported to be a substrate for OATP8 in oocytes.[7] In contrast, Yamaguchi *et al*.[8] showed that OATP8-overexpressing cells did not enhance paclitaxel uptake. Although the reason for this discrepancy remains unclear, it was possible that differences in the microenvironment, such as lipid composition, may have affected the substrate specificity of OATP8. Another research group have identified paclitaxel as a substrate of OAT2 that is expressed in *X. laevis* oocytes.[9] These findings suggested that paclitaxel and docetaxel were sensitive to one or several influx transporter(s), in addition to the P-gp efflux pump; however, it is necessary to evaluate key transporters of each hepatic uptake. Freshly isolated hepatocytes are widely accepted as the 'gold standard' for providing reliable data on drug uptake across the sinusoidal (basolateral) membrane.[10–12] Unlike in transfected cells, transporter-mediated uptake of paclitaxel and its derivatives has not been seen in fresh human and rodent hepatocytes. In rat hepatocytes, the transporters that are involved in hepatic uptake of anionic compounds can be classified into two families: the organic anion transporting polypeptides (Oatp1, 2 and 4) and the organic anion transporters (Oat 2 and 3). Among the known rat Oatps, Oatp4 has been characterized as the major organic anion uptake system of the Oatp-gene family.[13] As far as we know, rat Oat2 mediates the uptake of human OAT2 substrates except for tetracycline.[14–18] Therefore, fresh rat hepatocytes may clarify uptake properties of paclitaxel and its derivatives including prodrugs. Furthermore, slight amounts (1–5% of dose) of paclitaxel are produced from TAX-2′-Et in rat and human liver microsomes; however, rat serum provides rapid enzymatic hydrolysis of TAX-2′-Et.[6] The in-vitro disposition profile of TAX-2′-Et in human serum is similar to that observed in rabbit serum. The findings suggested that our preclinical GDEPT approach with TAX-2′-Et should be carried out using optimal animals as well as humans lacking carboxylesterase activity in the blood. This study will provide information on the detoxification pathways of P-gp-insensitive TAX-2′-Et in a GDEPT strategy with enzyme-expressing vector and paclitaxel prodrug.

In this study, we have characterized the uptake of paclitaxel and P-gp-insensitive TAX-2′-Et in freshly isolated and Oatp2 protein-induced hepatocytes in rats.

Materials and Methods

Chemicals

Paclitaxel, verapamil, indometacin, trypsin inhibitor (type II-S) and pregnenolone-16-α-carbonitrile (PCN) were purchased from Sigma Chemical Co. Ltd (St Louis, MO, USA). Rat tail collagenase I and rifampicin were obtained from Wako Pure Chemicals (Osaka, Japan). Cremophor EL was obtained from BASF (Mount Olive, NJ, USA). TAX-2′-Et was synthesized in our laboratory according to the method

reported by Ueda *et al.*[19] All other chemicals were of the highest grade commercially available.

Isolation of rat hepatocytes

Rat hepatocytes were harvested from male Wistar rats (250–280 g; Hamamatsu, Shizuoka, Japan) using a two-step in-situ collagenase perfusion method.[20] Briefly, rats were anaesthetized with sodium pentobarbital before cannulation of the portal vein. The liver was perfused with a Ca^{2+}-free oxygenated liver perfusion medium for 10 min, followed by addition of oxygenated collagenase buffer for 12 min at a flow rate of 20 ml/min. The digested liver was excised and hepatocytes were released into 30 ml ice-cold Dulbecco's modified Eagle's medium (DMEM). Cells were filtered through a nylon mesh and centrifuged at 500 rev/min for 30 s. The resulting cell pellet was washed three times with ice-cold DMEM and centrifuged at 500 rev/min for 30 s. Finally, the hepatocytes were resuspended in Krebs–Henseleit buffer (in mM: 118 NaCl, 23.8 $NaHCO_3$, 4.8 KCl, 1.0 KH_2PO_4, 1.2 $MgSO_4$, 12.5 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid (HEPES), 5 glucose and 1.5 $CaCl_2$ adjusted to pH 7.4) and all experiments were completed within 2 h after cell preparation. The viability of freshly isolated hepatocytes was measured using the Trypan blue exclusion test. Cells with a viability of greater than 90% were used for further study. Rats were used in accordance with ethical procedures following the guidelines for the care and use of laboratory animals issued by the Japanese government and Kinki University.

Induction of rat hepatic Oatp2 protein expression

PCN (75 mg/kg) was intraperitoneally administered to male Wistar rats (250–280 g) for four consecutive days. Vehicle-treated and untreated rats received corn oil (5 ml/kg per day) or saline (5 ml/kg per day), respectively. Hepatocytes were prepared according to the above method 24 h after the last dose.

Uptake studies in rat hepatocytes

Rat hepatocytes (2×10^6 cells) were prewarmed for 5 min in Krebs–Henseleit buffer (pH 7.4) at 37°C in the absence or presence of inhibitors (200 μM). The uptake studies were initiated by adding substrates (0.5 μM) to the cell suspension (2×10^6 cells). Kinetic analysis of the uptake of paclitaxel and TAX-2′-Et was performed in the substrate concentration range 0.12–2.34 μM because of their poor solubility. All inhibitors were dissolved in dimethyl sulfoxide (DMSO) and were subsequently diluted 1 : 200 in the incubation medium. Paclitaxel or TAX-2′-Et was solubilized with a mixture of Cremophor EL and ethanol (1 : 1, v/v) to yield a final concentration of 0.009% (19 nM). At the end of the incubation period (30 s), uptake was terminated by removing the medium, and the cells were immediately washed with ice-cold Krebs–Henseleit buffer containing 1% (2.1 μM) Cremophor EL and with 2 ml ice-cold Krebs–Henseleit buffer. To determine the amount of compounds adsorbed onto the surface of the cells, crude cell membrane fractions were prepared according to the method of Hosoya *et al.*[21] The membrane fractions incubated with paclitaxel or TAX-2′-Et at 37°C for 5 min were immediately washed with the detergent solution. The adsorptive amounts of paclitaxel and TAX-2′-Et were undetectable. Protein contents were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and bovine serum albumin as a standard. The uptake amounts of paclitaxel and TAX-2′-Et were normalized to the levels of total cellular protein.

Western blot analysis

Isolated rat hepatocytes were lysed in 300 μl buffer containing 1% Triton X-100 and 20 mM Tris-HCl (pH 7.6) supplemented with a mixture of protease inhibitors (Sigma Chemical Co. Ltd), 1 mM phenyl-methylsulfonyl fluoride and 1 M ethylenediamine-N,N,N′,N′-tetraacetic acid salts. The lysate was centrifuged at 5000*g* at 4°C for 5 min. Equal amounts (75 μg) of proteins from the supernatant were electrophoresed on SuperSept 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred electrophoretically to Immobilon membranes (Millipore, Bedford, MA, USA). Blots were probed overnight with a 1 : 1000 dilution of a polyclonal rabbit antibody to rat Oatp2 (OATP21S, Alpha Diagnostic International Inc., San Antonio, TX, USA) or with a 1 : 5000 dilution of an antibody to β-actin (Sigma-Aldrich). Membranes were washed in TBST (25 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20), containing 1% nonfat dry milk and were incubated with a 1 : 10 000 dilution of horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences, Buckingham, UK) or with a 1 : 2000 dilution of horseradish peroxidase–conjugated anti-mouse secondary antibody for 1 h. After three washes with TBST, bound antibody was detected by enhanced chemiluminescence (ECL plus, Amersham Biosciences, Buckingham, UK) and visualized on X-ray film.

High-performance liquid chromatography analysis

Cells were suspended in 35 mM ammonium acetate buffer (pH 5.0) containing medazepam as an internal standard. Paclitaxel and TAX-2′-Et were extracted in diethyl ether. After removal of the organic phase, the residue was dissolved in the mobile phase used for the high-performance liquid chromatography (HPLC) assay. The reconstituted solutions were injected onto an HPLC column for analysis. Paclitaxel and TAX-2′-Et were separated using a Cosmosil–MS 5C8 column (5 μm, 4.6 × 250 mm, Nacalai Tesque Co., Kyoto, Japan). HPLC analysis was performed on a system equipped with a Shimadzu SPD-10A, a UV detector, a Shimadzu LC-10A pump and a Shimadzu C-R4A chromatopac integrator. Detection was performed at 230 nm. A mobile phase of acetonitrile and 35 mM ammonium acetate buffer (pH 5.0) at a 1 : 1 (v/v) ratio was used at a flow rate of 1.0 ml/min.

Determination of the kinetic parameters and statistical analysis

Uptake of paclitaxel and TAX-2′-Et by rat hepatocytes increased linearly against time up to 1 min (data not shown). Thus, the initial uptake velocity was calculated by linear regression using the data point at 30 s. Initial uptake rates were normalized to protein content. The kinetic parameters K_m (Michaelis constant) and V_{max} (maximum uptake rate) were calculated using the Michaelis–Menten equation and the nonlinear regression program, MULTI.[22]

To determine statistically significant differences among the experimental groups, the nonparametric Kruskal–Wallis test was used for multiple comparisons, and post-hoc Dunn's test for two-group comparison when appropriate. A P value of less than 0.05 was termed significant.

Results

Uptake of paclitaxel and TAX-2′-Et into untreated rat hepatocytes

The hepatic uptake of paclitaxel and TAX-2′-Et was examined in rat hepatocyte suspensions (Figure 2). Paclitaxel uptake was concentration-dependent with a K_m value of 1.5 ± 0.3 μM and a V_{max} value of 4.2 ± 0.4 pmol/s per mg protein. TAX-2′-Et uptake also showed saturation with a K_m value of 1.7 ± 0.4 μM and a V_{max} value of 2.3 ± 0.3 pmol/s per mg protein. The intrinsic transport capacity (V_{max}/K_m) was two-fold higher for paclitaxel uptake than for TAX-2′-Et uptake.

Inhibitory effects of verapamil, rifampicin and indometacin on paclitaxel and TAX-2′-Et uptake into untreated rat hepatocytes

To characterize the concentration-dependent uptake of paclitaxel and TAX-2′-Et, we evaluated the inhibitory effects of verapamil (an inhibitor of P-gp, rat Oatp1, and rat Oatp2), rifampicin (a potent inhibitor of rat Oatp2), and indometacin (a representative inhibitor of rat Oatp1 and Oat2) at appropriate concentrations (Figure 3). In isolated rat hepatocytes, these compounds are known to effectively inhibit carrier-mediated uptake systems at concentrations of 100 μM or more.[23–25] The uptake of paclitaxel and TAX-2′-Et was significantly inhibited by rifampicin, but not by indometacin. Rifampicin or indometacin (200 μM) did not induce leakage of lactate dehydrogenase even after incubation for 30 min with the hepatocyte suspensions (data not shown).

Uptake of paclitaxel and TAX-2′-Et into PCN-induced hepatocytes

To further characterize the rifampicin-sensitive uptake of paclitaxel and TAX-2′-Et, Oatp2 protein levels were

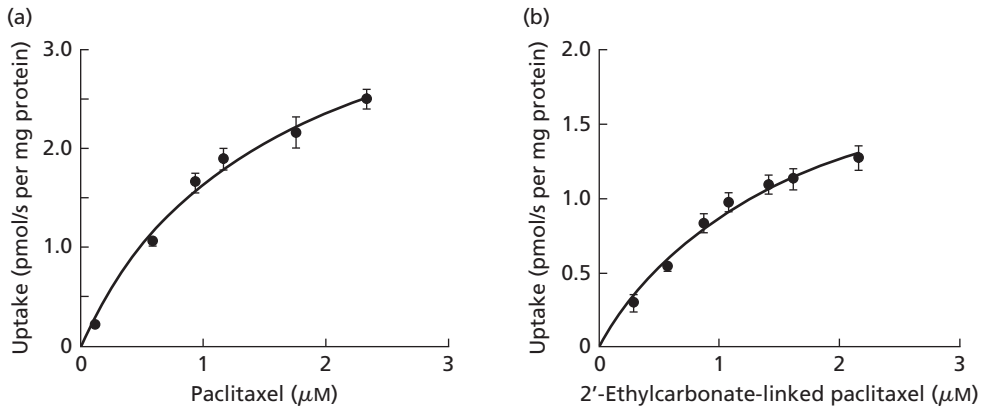

Figure 2 Uptake of paclitaxel and 2′-ethylcarbonate-linked paclitaxel into freshly isolated rat hepatocytes. Paclitaxel (a) and 2′-ethylcarbonate-linked paclitaxel (b) were incubated at 37°C for 30 s. The solid line represents the least-squares fit of the data to the Michaelis–Menten equation. Data are expressed as the mean ± SD of three to four uptake experiments.

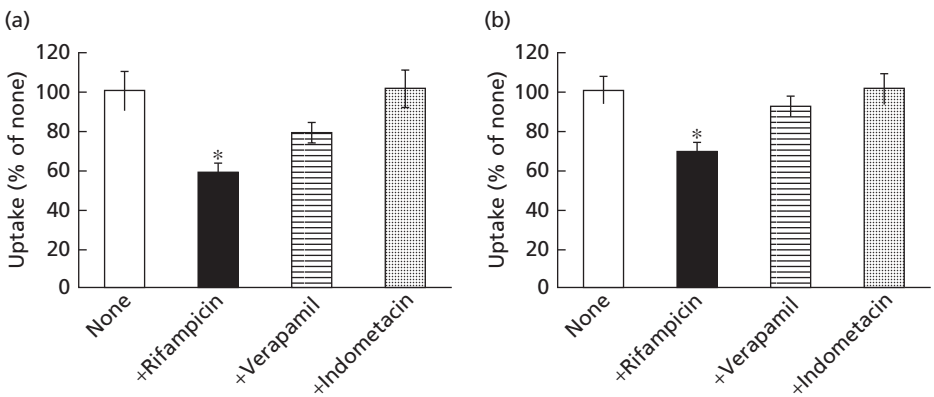

Figure 3 Inhibitory effects of rifampicin, verapamil and indometacin on the uptake of paclitaxel and 2′-ethylcarbonate-linked paclitaxel into untreated hepatocytes. Rat hepatocyte suspensions were exposed for 30 s to 0.5 μM paclitaxel (a) or 2′-ethylcarbonate-linked paclitaxel (b) in the absence or presence of the various inhibitors (200 μM). The results are given with the standard deviation ($n = 4$). *$P < 0.05$ compared with substrate uptake in the absence of inhibitor (None).

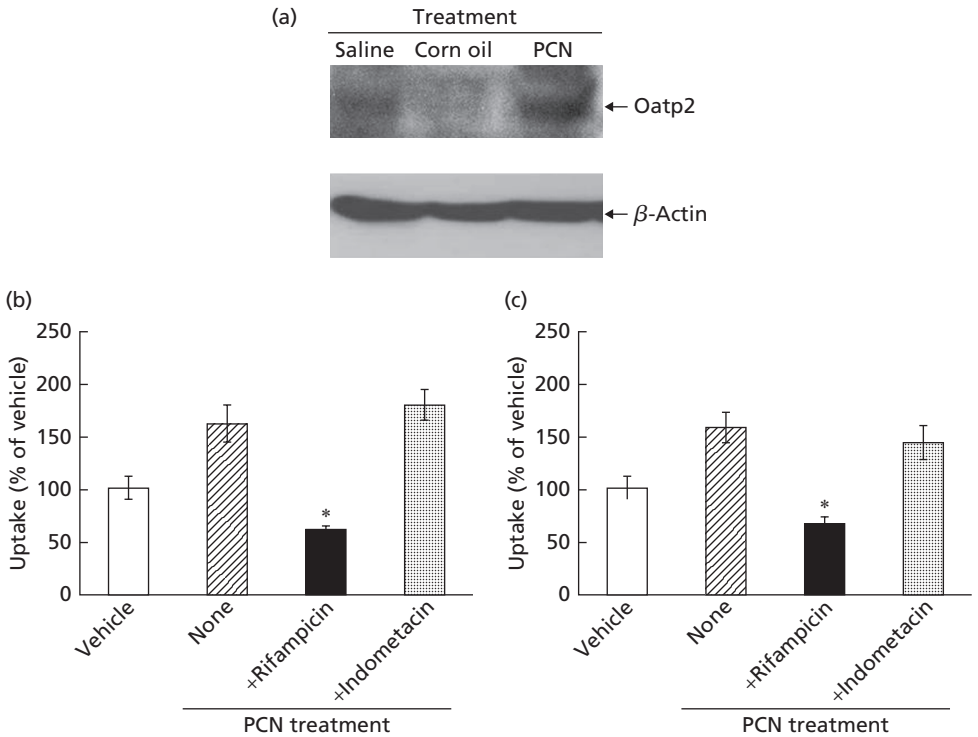

Figure 4 Uptake of paclitaxel and 2′-ethylcarbonate-linked paclitaxel into pregnenolone-16-α-carbonitrile-induced rat hepatocytes. (a) Pregnenolone-16-α-carbonitrile (PCN) (75 mg/kg per day) was intraperitoneally administered in corn oil (5 ml/kg per day) for four consecutive days. Vehicle-treated control and untreated rats received corn oil or saline, respectively. Crude membrane fractions (75 μg) prepared from saline-, PCN- or vehicle-treated rat hepatocytes were loaded and separated by SDS-PAGE. Paclitaxel (b) or 2′-ethylcarbonate-linked paclitaxel (c) was incubated with PCN-induced hepatocytes for 30 s in the absence or presence of rifampicin and indometacin. The results are given with the standard deviation ($n = 4$). *$P < 0.05$ compared with substrate uptake in PCN-treated hepatocytes (None).

upregulated by intraperitoneal administration of PCN into rats. The expression level of Oatp2 in crude membranes prepared from freshly isolated rat hepatocytes was investigated by Western blot analysis (Figure 4a). Corn oil treatment did not alter the expression levels of Oatp2 protein when compared with saline. Treatment of rats with PCN increased hepatic Oatp2 protein levels. PCN-treated hepatocytes enhanced the uptake of paclitaxel and TAX-2′-Et when compared with corn oil-treated hepatocytes (Figure 4b and c). The increased uptake of paclitaxel and TAX-2′-Et was sensitive to rifampicin, but not indometacin.

Discussion

It is possible to predict the in-vivo elimination rate of drugs based on their initial uptake rate in isolated hepatocytes and the number of hepatocytes per gram of liver.[26,27] Conventional and sandwich cultures of rat hepatocytes show dramatically decreased protein levels of Oatp and Oat isoforms with increased culture periods.[28,29] Compared with cultured rat hepatocytes, greater Oatp-mediated uptake of taurocholate (a substrate of Oatp1 and 2) is maintained in fresh hepatocyte suspensions.[29] Treiber *et al.*[30] also showed that cholecystokinin peptide (CCK-8, an inhibitor of Oatp4) significantly reduced the active uptake of bosentan in freshly prepared rat hepatocytes. Oatp4 exhibited the highest amino acid sequence homology with OATP8 (66%).[13] It was liver-specific and was the most highly expressed of the known rat Oatps.[31] To characterize the saturated uptake of paclitaxel and TAX-2′-Et in fresh rat hepatocyte suspensions, we examined the inhibitory effect of CCK-8, however, CCK-8 treatment did not alter paclitaxel uptake (data not shown). Therefore, the saturated uptake of paclitaxel and TAX-2′-Et probably relied on influx transporters other than Oatp4.

Rat Oat2 is abundantly expressed in the liver and exhibited 79% homology with human OAT2.[14,32,33] Sekine *et al.*[14] examined the uptake activity of rat Oat2 using a *X. laevis* expressing system and demonstrated that rat Oat2 transported several organic anions, such as salicylate, α-ketoglutarate, methotrexate, prostaglandin and *p*-aminohippurate. It is also known that indometacin is a substrate for rat Oat2 and Oat1, but not for Oat3.[15,34] Its affinity for rat Oat2 was higher than that for rat Oat1.[15] Among the known compounds, indometacin is the substrate with the highest affinity in rat Oat2-expressing porcine kidney epithelial (LLC-PK1) cells (K_m value: 0.4 μM).[15] In isolated rat hepatocyte suspensions, indometacin (100 μM) inhibited Y-700 uptake with a reduction of 50%.[25] Furthermore, Shitara *et al.*[35] showed that indometacin (100 μM) dramatically decreased rat Oatp1-mediated uptake of oestradiol 17β-D-glucuronide in cDNA-transfected LLC-PK1 cells. In this study, indometacin was added to rat hepatocyte suspensions at the higher concentration of 200 μM; however, the uptake of paclitaxel and TAX-2′-Et was not altered (Figure 3). Therefore, it seemed that the

saturated uptake of paclitaxel and TAX-2′-Et was insensitive to rat Oat1, Oat2, and Oatp1 transporters, in addition to the Oatp4 transporter.

The uptake of digoxin, a typical neutral substrate of the OATP8 transporter, is not mediated by rat Oatp4, but has a high affinity for rat hepatic Oatp2, which shows only 45% amino acid sequence homology with human OATP8.[35] Rat Oatp2 is predominantly expressed in the liver and transports many structurally unrelated compounds, including anions, cations and neutral compounds.[35–38] It is also known that rat Oatp2 shares overlapping substrate specificities with OATP8.[35] Rat hepatocytes functionally maintain organic cation transporter (rat, Oct; human, OCT) 1 and taurocholate cotransporting polypeptide (rat, ntcp; human, NTCP).[28,39] In *X. laevis* oocytes, some human solute carrier subfamily members (OAT1, OAT3, OCT1 and NTCP) do not facilitate paclitaxel accumulation.[40] Judging from these findings and our indometacin-insensitive transport, we focused on the contribution of Oatp2 and examined the inhibitory effect of rifampicin. The uptake of paclitaxel and TAX-2′-Et was reduced to 55 and 72% of the control, respectively, suggesting that Oatp2 was a candidate transporter for the saturated uptake of paclitaxel and TAX-2′-Et.

Chemicals can induce uptake and export transport proteins in the liver and intestine. PCN increased mouse and rat hepatic Oatp2 mRNA contents.[41,42] Rat hepatic Oatp2, but not Oatp1, is upregulated 20-fold at the protein level by PCN and 2.3-fold by phenobarbital.[41] Phenobarbital-treated rat hepatocyte suspensions moderately increased the uptake of cardiac glycosides (digoxin, digitoxin, and ouabain), however, PCN-treated rat hepatocyte suspensions significantly increased ouabain uptake.[41] In contrast, Matheny *et al*.[43] showed that mouse hepatic P-gp expression was not induced by PCN. As shown in Figure 4a, PCN treatment dramatically upregulated the protein levels of Oatp2 expressed in isolated rat hepatocytes. Therefore, Oatp2-mediated uptake of paclitaxel and TAX-2′-Et was evaluated in PCN-treated rat hepatocytes. PCN-treated hepatocytes showed a 1.6-fold greater uptake of paclitaxel and TAX-2′-Et than corn oil (vehicle)-treated hepatocytes, and the uptake of paclitaxel and TAX-2′-Et was significantly inhibited by rifampicin (Figure 4b and c). In rats and mice, it is not yet known whether PCN regulates expression levels of influx transporters except for an organic anion transporter 1. Since human OAT2-expressing oocytes accelerated cellular uptake of paclitaxel, we reconfirmed the inhibitory effect of indometacin in PCN-treated hepatocytes. Consequently, the results strongly suggested that both paclitaxel and TAX-2′-Et were a substrate of the rat hepatic Oatp2 transporter.

Conclusions

The concentration-dependent uptake of paclitaxel and P-gp-insensitive TAX-2′-Et was associated with rat Oatp2 transporter activity, but not Oatp1, 4, Oat1, or Oat2. Our findings provide an important step towards identifying a key transporter in hepatic detoxification of paclitaxel and TAX-2′-Et in small animals.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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