JPP
Journal of
Pharmacy and Pharmacology

JPP 2005, 57: 1305–1311
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Received April 6, 2005
Accepted June 7, 2005
DOI 10.1211/jpp.57.10.0009
ISSN 0022-3573

Transporter-mediated influx and efflux mechanisms of pitavastatin, a new inhibitor of HMG-CoA reductase

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Abstract

The purpose of this study was to gain a better understanding of the transport mechanism of pitavastatin, a novel synthetic HMG-CoA reductase inhibitor. Experiments were performed using oocytes of *Xenopus laevis* expressing several solute carrier (SLC) transporters and recombinant membrane vesicles expressing several human ABC transporters. The acid form of pitavastatin was shown to be a substrate for human OATP1, OATP2, OATP8, OAT3 and NTCP, and for rat Oatp1 and Oatp4 with relatively low K_m values. In contrast, these SLC transporters were not involved in the uptake of the lactone form. A significant stimulatory effect was exhibited by pitavastatin lactone, while the acid form did not exhibit ATPase hydrolysis of P-glycoprotein. In the case of breast cancer resistant protein (BCRP), the acid form of pitavastatin is a substrate, whereas the lactone form is not. Taking these results into consideration, several SLC and ABC transporters were identified as critical to the distribution and excretion of pitavastatin in the body. This study showed, for the first time, that acid and lactone forms of pitavastatin differ in substrate activity towards uptake and efflux transporters. These results will potentially contribute to the differences in the pharmacokinetic profiles of pitavastatin.

Introduction

The processes involved in metabolic biotransformation, especially those mediated by cytochrome P450s (CYPs), play a major role in metabolic fate of drugs. Recently, it has been recognized that additional processes such as membrane-bound transport systems may be similarly important (Ambudkar et al 1999; Shitara et al 2005). In particular, the hepatic and renal transport system in the sinusoidal and basolateral membrane is responsible for the clearance of various drugs from the systemic circulation.

Pitavastatin is a novel synthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (statin) and has a persistent effect on serum lipids (Aoki et al 2002). Its efficacy and safety have been confirmed in clinical practice (Saito et al 2002; Kajinami et al 2003). ^{14}C-Pitavastatin was selectively distributed to the rat liver and maximum radioactivity was approximately 54 times that in plasma (Kimata et al 1998). Recently, we found that multispecific anion transporters and organic anion transporting polypeptide-2 (OATP2) were critically involved in the uptake of pitavastatin into the liver in rats and man, respectively (Shimada et al 2003; Fujino et al 2004a; Hirano et al 2004). Although the mechanism of excretion has not been fully elucidated, canalicular multispecific organic anion transporter (Mrp2) and P-glycoprotein (Pgp) did not play a major role in the transport of pitavastatin (Fujino et al 2002). Since pravastatin, rosuvastatin and pitavastatin cannot undergo metabolism via CYPs, the frequency of drug–drug interaction was believed to be low. However, remarkable increases in AUCs were reported after the co-administration of ciclosporin with these statins (Park et al 2002; Hasunuma et al 2003; Simonson et al 2004). OATP2 accepts these statins as substrates and OATP2-mediated uptake of statins was inhibited by ciclosporin (Mück 2000; Nakai et al 2001; Shitara et al 2003; Fujino et al 2004a). From these results, when considering the drug–drug interaction of statins, one should pay attention to the characterization of the transporters.

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Acknowledgment: The authors are grateful to Prof. Yuichi Sugiyama, University of Tokyo, and Dr Yoshihisa Shitara, Chiba University, for invaluable suggestions.

Pitavastatin is administered in an acid form, although the area under the concentration time curve (AUC) of the lactone form as a major metabolite was similar in clinical studies (Kojima et al 1999). Recent studies have additionally demonstrated that uridine 5′-diphosphate (UDP) glucuronosyl transferase (UGT) is critically involved in the lactonization of pitavastatin in man and animals (Fujino et al 2003; Yamada et al 2003). On the other hand, CYP2C9 is involved in the metabolism of the acid form. In contrast, CYP2C9 is not involved in the metabolism of the lactone form and CYP3A4 is closely involved (Fujino et al 2004b). These results demonstrate that the acid and lactone forms have different metabolic properties. Therefore, when considering the transport mechanism of pitavastatin, one should focus on the transporter profiles of both forms.

To date, only the acid form has been studied regarding membrane-bound transport systems. To gain a better understanding of the transporter profiles of pitavastatin, we performed several experiments using *Xenopus* oocytes expressing the solute carrier (SLC) transporter and recombinant membrane vesicles expressing human ATP-binding cassette (ABC) transporters, with the goal of characterizing the transporter profiles of pitavastatin.

Materials and Methods

Chemicals and reagents

Pitavastatin and pitavastatin lactone were synthesized by Nissan Chemical Industries (Chiba, Japan) (Figure 1). Fluorobenzene-U-^{14}C pitavastatin was synthesized by Amersham Bioscience Co. (Little Chalfont, UK). ^{14}C-Pitavastatin lactone was produced according to our previous report (Yamada et al 2003). The specific radioactivity was 3.46 MBq (mg)$^{-1}$ and the radiochemical purity was more than 99% during the experimental period. ^{3}H-Estrone-3-sulfate, ^{3}H-taurocholic acid, ^{14}C-tetraethylammonium, ^{14}C-*p*-aminohippuric acid and ^{3}H-estradiol-17β-D-glucuronide were purchased from Amersham Bioscience Co. or NEN Life Science Products (Boston, MA). The radiochemical purity of these labelled compounds was more than 98%. All other chemicals and reagents used were commercially available and of guaranteed purity.

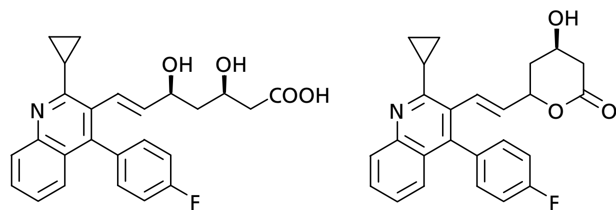

Figure 1 Chemical structures of pitavastatin (left) and its lactone form (right).

Transporters

The transportocytes of *Xenopus laevis* (African claw frog) expressing human OATP1 (organic anion transporting polypeptide-1: *SLC21A3*), OATP2 (organic anion transporting polypeptide-2: *SLC21A6*), OATP8 (organic anion transporting peptide-8: *SLC21A8*), OAT1 (organic anion transporter-1: *SLC22A6*), OAT3 (organic anion transporter-3: *SLC22A8*), OCT1 (organic cation transporter-1: *SLC22A1*), NTCP (Na-taurocholic acid cotransport protein: *SLC10A1*), rat Oatp1 (organic anion transporting polypeptide-1: *Slc21a1*) and Oatp4 (organic anion transporting polypeptide-4: *Slc21a10*) were purchased from BD-GENTEST (Woburn, MA). The recombinant membrane vesicles derived from baculovirus-expressing human Pgp and MRP2, and derived from mammalian species expressing human BCRP, were purchased from BD-GENTEST and SOLVO Co. Ltd (Budapest, Hungary), respectively.

Assessment of pitavastatin uptake by oocytes

Ten oocytes per experimental condition were placed in a 5-mL test tube with a small volume of Barth's solution. The Barth's solution was removed and replaced with 300 μL of Barth's solution containing either ^{14}C-pitavastatin or ^{14}C-pitavastatin lactone at a concentration of 5 μM. The oocytes were incubated at 20°C for 120 min. After the incubation, the solution was aspirated and the oocytes were washed 3 times by the addition of 2.5 mL of ice-cold Barth's solution to remove the remaining labelled substrate and to prevent further uptake. Individual oocytes were placed into vials containing 150 μL of 2% sodium dodecyl sulfate and allowed to lyse. This was followed by the addition of 5 mL of scintillation fluid. Water-injected oocytes were used as controls. ^{3}H-Estrone-3-sulfate was used as a positive control for OATP1-, OATP2-, OAT3-, Oatp1- and Oatp4-mediated uptake experiments, respectively. In other studies, ^{3}H-taurocholic acid, ^{14}C-tetraethylammonium, ^{14}C-*p*-aminohippuric acid and ^{3}H-estradiol-17β-D-glucuronide were used as a positive control for NTCP-, OCT1-, OAT1- and OATP8-mediated uptake experiments, respectively. The ^{14}C and ^{3}H content was then measured by scintillation spectrophotometry.

To measure the specific transporter-mediated uptake of pitavastatin, the uptake of pitavastatin was measured in parallel in transporter-expressing oocytes and control oocytes for each substrate concentration. The uptake into control oocytes was then subtracted from the uptake into transporter-expressing oocytes. The kinetic parameters were calculated according to equation 1:

$$V_{o,O} = V_{o,max} \cdot S/(K_m + S) \qquad (1)$$

Where $V_{o,O}$ is the initial uptake rate (pmol h^{-1} per oocyte), $V_{o,max}$ is the maximal uptake rate (pmol h^{-1} per oocyte), S is the pitavastatin concentration (μM) in the medium and K_m is the Michaelis constant (μM). The uptake data were fitted to the above equation by a non-

linear least-squares method at concentrations of 1–100 μM of the acid form.

Stimulation of ATPase hydrolysis

The ability of compounds to stimulate ATP hydrolysis was examined using the membrane-vesicle-expressing human Pgp, MRP2 and BCRP. The method used to determine the drug-simulated ATPase activity was described in our previous report (Fujino et al 2002). Briefly, ATP assays were conducted in 96-well microtitre plates using human Pgp membrane at concentrations of 1–120 μM of the acid form and 1–100 μM of the lactone form, respectively. The initial assay volume was 0.06 mL and the incubation time was 20 min. Incubations were conducted in duplicate and the drug-stimulated ATPase activity was determined as the difference between the amount of inorganic phosphate released from ATP in the absence and presence of vanadate. The assay was stopped by the addition of 0.2 mL of sodium dodecyl sulfate/developing reagent and the response was measured as absorbance at 620 nm. Potassium phosphate standards were prepared in each plate and verapamil served as a positive control of Pgp substrate. Kinetic parameters (apparent K_m) were estimated by fitting the drug concentrations and ATP hydrolysis activity into the Michaelis–Menten equation. MRP2 and BCRC were evaluated in the same manner as Pgp. In addition, verapamil, probenecid and sulfasalazine were used as a positive control for Pgp-, MRP2- and BCRP-mediated ATPase experiments, respectively.

Statistical analysis

Statistical analysis of the time courses of uptake of ^{14}C-pitavastatin between transporter-expressing and control oocytes was performed using Friedman's test. Also, the statistical analysis of the uptake ratios on several transporters were analysed by the Kruskal–Wallis test. For the difference between the acid form and lactone form, the Mann–Whitney *U*-test was used. In all cases, post-hoc comparisons of the means of individual groups were performed using Dunn's test. $P < 0.05$ denoted significance in all cases.

Results

Uptake of pitavastatin into SLC transporter-expressing oocytes

The time course of the uptake of ^{14}C-pitavastatin into human OATP2- or OATP8-expressing oocytes and control oocytes is shown in Figure 2. The data show that the acid form of pitavastatin is a good substrate for OATP2 and OATP8. The amount of the acid form taken up into human OATP2- and OATP8-expressing oocytes was about two- and three-fold those in water-injected oocytes, respectively. An initial experiment showed that the rate of transport was linear within a 60-min incubation period.

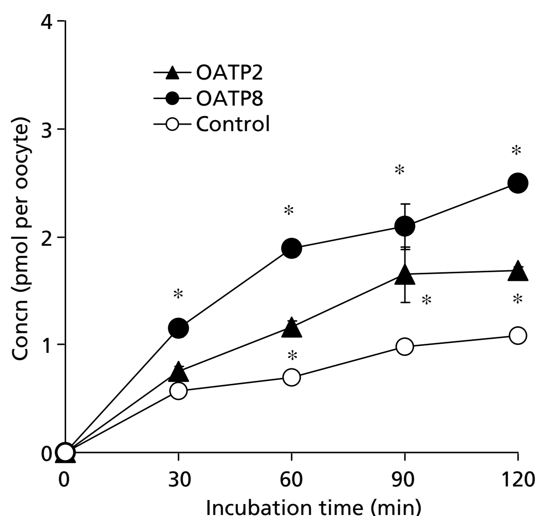

Figure 2 Time course of ^{14}C-pitavastatin uptake by OATP2- and OATP8-expressing oocytes. Each point represents the mean ± s.e. of ten oocytes. $^{*}P < 0.05$ compared with control oocytes.

Therefore, 60 min was used as the duration of subsequent experiments. The uptake of the acid form into other transporter-expressing oocytes is shown in Figure 3. The uptake was observed in human OATP1-, OATP2-, OATP8-, OAT3-, and NTCP-, and rat Oatp1- and Oatp4-expressing oocytes. These results suggested that the acid form of pitavastatin could be a substrate of these transporters, whereas OAT1 and OCT1 were not. In contrast to the acid form, the uptake of the lactone form was negligible in all oocytes examined.

Kinetic analysis

The concentration dependency of the uptake of the acid form of pitavastatin was observed in oocytes over a concentration range of 1–100 μM. The obtained apparent K_m values were 3.4 μM for human OATP1, 3.9 μM for OATP2, 3.8 μM for OATP8, 15.1 μM for NTCP and 3.3 μM for OAT3 in the human transporter (Table 1). In the case of the rat transporter, the obtained K_m values were 7.9 μM for Oatp1 and 4.8 μM for Oatp4. In addition, the obtained K_m values of OATP2 and OATP8 were almost the same as in previous reports (Fujino et al 2004a; Hirano et al 2004).

Activation of ATPase in ABC transporter membranes

Plots of ATP hydrolysis as a function of drug concentrations for pitavastatin are shown in Figure 4 and the estimated apparent kinetic parameters are listed in Table 2. A significant stimulatory effect was exhibited by pitavastatin lactone, while the acid form did not exhibit ATPase hydrolysis. These results suggest that the lactone form was a substrate of Pgp, whereas the acid form was not. The apparent K_m value of the lactone

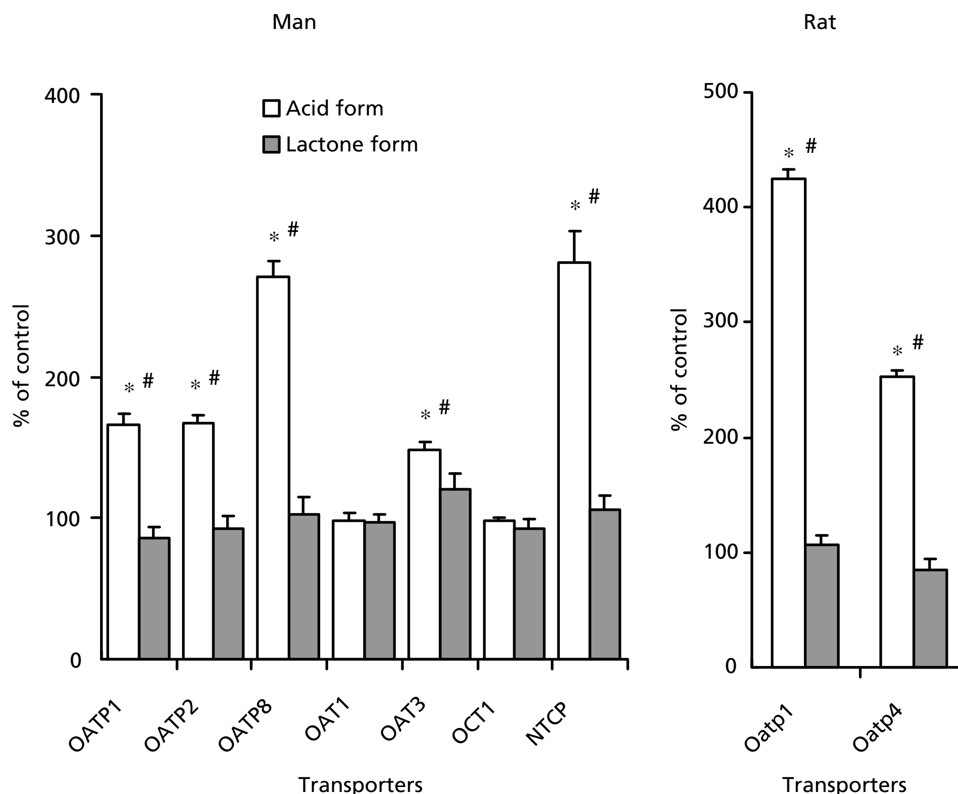

Figure 3 Uptake ratios of ^{14}C-pitavastatin acid form and lactone form in SLC transporter-expressing oocytes after incubation for 60 min. Each point represents the mean and s.e. of ten oocytes. $*P < 0.05$ compared with control oocytes (Kruskal–Wallis test); $\#P < 0.05$ compared with lactone form (Mann–Whitney *U*-test).

Table 1 Kinetic parameters for the uptake of ^{14}C-pitavastatin by SCL transporter-expressing oocytes

Transporter	K_m (μM)	Vmax (pmol h^{-1} per oocyte)
Human OATP1	3.4 ± 0.3	2.84 ± 0.12
Human OATP2	3.6 ± 1.2	0.90 ± 0.22
Human OATP8	3.8 ± 0.3	2.79 ± 0.40
Human NTCP	15.1 ± 4.7	16.33 ± 3.96
Human OAT3	3.3 ± 1.1	0.76 ± 0.25
Rat Oatp1	7.9 ± 2.8	10.44 ± 2.20
Rat Oatp4	4.8 ± 0.5	5.29 ± 0.43

Data represent the mean ± s.d. of three experiments.

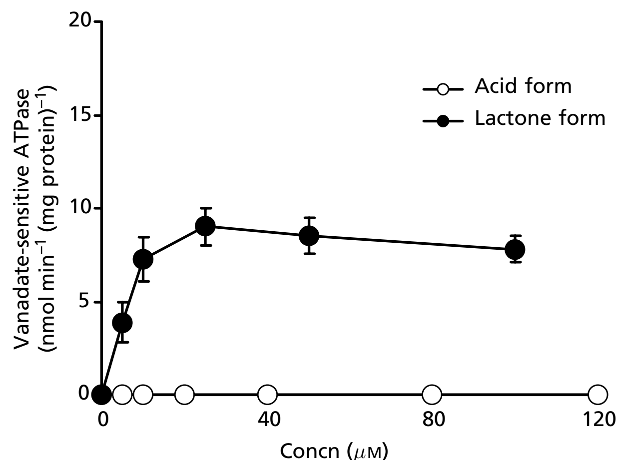

Figure 4 Concentration response for the stimulation of Pgp-mediated ATPase activity by pitavastatin acid form (open circles) and lactone form (closed circles). Each point represents the mean ± s.e. of triplicate determinations.

form, demonstrated by a Lineweaver–Burk plot, was 5.1 μM. In the case of MRP2 vesicle membranes, the acid form and lactone form did not have a significant stimulatory effect, demonstrating that MRP2 did not play a role for the distribution of either form. Although the apparent K_m value was not calculated, a significant stimulatory effect on ATPase hydrolysis was exhibited by the acid form in BCRP vesicle membranes. In contrast, ATPase hydrolysis was not exhibited in the presence of the lactone form.

Discussion

Since the liver is a major site of the clearance, as well as a pharmacological target, of statins, it is essential to clarify

Table 2 Kinetic parameters for pitavastatin acid form and lactone form by several ABC transporters

Transporter	Acid form		Lactone form	
	ATPase hydrolysis	K_m (μM)	ATPase hydrolysis	K_m (μM)
MDR1	Negative	—	Positive	5.1 ± 1.2
MRP2	Negative	—	Negative	—
BCRP	Positive	n.c.	Negative	—

Data represent the mean ± s.d. of three determinations. —, not performed; n.c., not calculated.

the transporter profiles of pitavastatin to predict the pharmacological effects and drug–drug interactions. Previously, the uptake of pitavastatin was investigated in cryopreserved human hepatocytes and the hepatic uptake reached saturation with an apparent K_m of 3.0 μM (Fujino et al 2004a). In this study, we showed that pitavastatin is a substrate for OATP2, OATP8 and NTCP, which are localized on the sinusoidal membrane of human hepatocytes, with an apparent K_m of 3.9, 3.8 and 15.1 μM, respectively. OAT1 and OCT1, however, which are also localized on the sinusoidal membrane, were not involved. In the clinical setting, the maximum plasma concentration (Cmax) of pitavastatin is approximately 0.08 μM (after a single oral dose of 2 mg) (Fujino et al 1999) and the Cmax is much lower than the K_m values obtained in this study, suggesting that transporters are critically involved in the hepatic uptake of pitavastatin over the therapeutic range. Moreover, pitavastatin was shown to be a substrate for rat Oatp1 and Oatp4, which are localized to rat hepatocytes, with an apparent Km of 7.9 and 4.8 μM, respectively. Oatp4 has the highest level of homology to human OATP2 and is selectively expressed in the rat liver (Cattori et al 2000). Taking these results into consideration, as well as those in man, Oatp families play a major role in the uptake of pitavastatin in rats. In contrast to the acid form, the lactone form was not found to be an OATP2 substrate. In the clinical setting, the AUC of pitavastatin was increased 4.5 fold in healthy subjects after co-administration of ciclosporin as a potent inhibitor of OATP2 (Hasunuma et al 2003, Fujino et al 2004a). However, the plasma concentration of the lactone form was not affected, indicating that the transporter properties of pitavastatin were suitable for the clinical use of this drug. In addition, the influence of co-administered drugs on the uptake of pitavastatin has not been reported using human hepatocytes; however, a significant effect can be expected. Further study is required to clarify the relationship between transporter-mediated drug–drug interactions and the pharmacokinetics of pitavastatin.

Previously, the urinary excretion rate of unchanged pitavastatin was reported to be 2–4% of the dose in man (Fujino et al 1999), although the mechanism of excretion was not fully elucidated. Recently, Takeda et al (2004) have reported that OAT3, which is localized to the proximal tubule, plays a major role in the urinary excretion of pravastatin. In our study, pitavastatin was shown to be a substrate for OAT3 with a K_m of 3.3 μM. Thus, it was suggested that human OAT3 is partly associated with the tubular secretion of pitavastatin.

Brain capillary endothelial cells are characterized by the expression of several transporters, such as Oat3, Oatp2 and Mdr1 (Kusuhara & Sugiyama 2001; Sugiyama et al 2001; Ohtsuki et al 2002). These transporters facilitate the elimination of endogenous compounds from the central nervous system (CNS) across the blood–brain barrier (BBB), providing the barrier function between the blood and the brain. Although pitavastatin is more lipophilic than pravastatin, its brain-to-plasma concentration ratio has been reported to be lower than that of pravastatin (Komai et al 1992; Kimata et al 1998). Recently, Kikuchi et al (2004) have reported that pravastatin and pitavastatin undergo efflux from the rat brain into blood across the BBB and that the intrinsic efflux clearance of pitavastatin is 6-fold greater than that of pravastatin. Also, at least two transporters, rat Oat3 and Oatp2, are critically involved in the efflux processes of these statins (Kikuchi et al 2004). In our study, pitavastatin was shown to be a substrate for OAT3 and OATP1. OAT3 is expressed in the human brain, and the expression of OAT3 at the choroids plexus, acting as a barrier between the blood and the cerebrospinal fluid, has been reported (Alebouyeh et al 2003). Also, OATP1 has the highest level of homology to rat Oatp2 and is expressed at the BBB (Gao et al 2000). Taking these findings into consideration, it is possible that OATP1 and OAT3 play an important role in the efflux of pitavastatin across the barriers of the CNS.

Biliary excretion is an important pathway in the elimination of pitavastatin and its metabolites (Kojima et al 2001). Although the process by which pitavastatin is effluxed has not been fully elucidated, no critical roles for Mrp2 and Pgp were found (Fujino et al 2002). In this study, we found that the acid form of pitavastatin is not a Pgp substrate, whereas the lactone form is. Also, the lactone form is not a substrate of BCRP, but BCRP was closely involved with the acid form. These results demonstrate that the acid and lactone forms exhibit different ABC transporter properties. Consistent with our results, several researchers have reported that the lactone forms of simvastatin, lovastatin, atorvastatin and rosuvastatin are, but the acid forms are not, substrates for Pgp (Huang et al 2003; Chen et al 2005). Taking these results into consideration, when considering the transport mechanism of statins, one should pay attention to the transporter profiles of both forms. Although this study has shown that pitavastatin lactone is a Pgp substrate, the likelihood of increasing the systemic concentration of pitavastatin lactone, and consequently significant interaction when pitavastatin is co-administered with a known Pgp inhibitor such as grapefruit juice or ciclosporin, is minimal (Hasunuma et al 2003; Tomlinson 2003).

Genetic polymorphism of drug transporters can result in variations in the pharmacological and toxicological effects of drugs, leading to inter-individual differences in response. Recently, a number of single nucleotide polymorphisms (SNPs) for the OATP2 gene have been

identified, and most of these SNPs were associated with significantly reduced transporter activity (Tirona et al 2001; Iwai et al 2004). Regarding pravastatin, Japanese subjects with the OATP2*15 allele showed reduced clearance compared with subjects with the OATP2*1b allele (Nishizato et al 2003). The reduced hepatic uptake due to this gene polymorphism was associated with a lower hepatic concentration and a higher plasma concentration, resulting in attenuation of the lipid-lowering effect or an increase in the risk of serious side effects of statins (Tachibana-Iimori et al 2004). The influence of genetic polymorphisms for OATP2 on the plasma concentration of pitavastatin has not been reported yet, although a significant effect can be expected. Further study is required to identify how much of the pharmacokinetic variability is conferred by OATP2 alleles and to clarify the relationship between polymorphisms in the OATP2 gene and the pharmacokinetics of pitavastatin. Moreover, pharmacokinetic studies of rosuvastatin have demonstrated an approximate 2-fold elevation in median exposure (AUC) in Asian subjects when compared with a Caucasian control group (FDA Public Health Advisory on Crestor 2005). This increase should be considered when deciding on doses of rosuvastatin for Asian patients. Although the ethnic difference between Asians and Caucasians cannot be fully explained, genetic polymorphisms of transporters might contribute partly to the increase in the plasma concentration of rosuvastatin.

Conclusion

This study showed for the first time that the acid and lactone forms of pitavastatin had differential activity towards SLC and ABC transporters. The process of pitavastatin elimination in the human liver is shown in Figure 5.

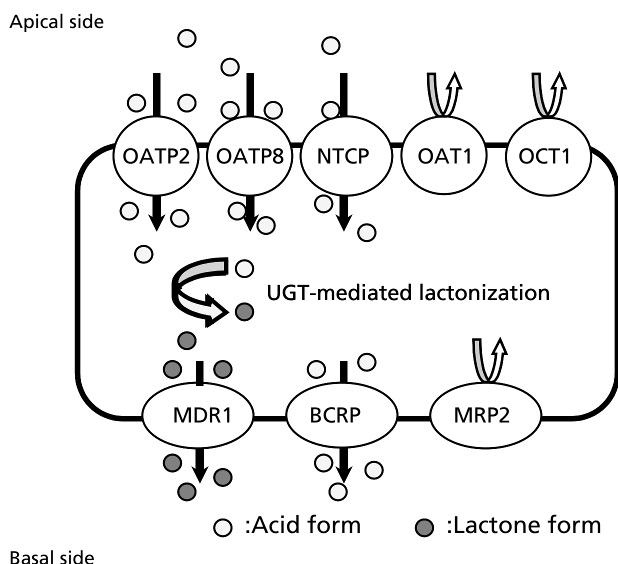

Figure 5 Processes of pitavastatin elimination in the liver. Pitavastatin is taken up into hepatocytes via the transporter, and undergoes lactonization and biliary excretion.

Our results suggest that the metabolic and transporter profiles of pitavastatin in man are complex, involving acid/lactone interconversion. Both forms of pitavastatin are observed in-vivo following oral administration; the relative composition of the acid and lactone forms may ultimately contribute to the differences in pharmacokinetic profiles and will potentially influence transporter-mediated drug–drug interactions.

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