Regular Article

Long-lasting Inhibitory Effects of Cyclosporin A, but Not Tacrolimus, on OATP1B1- and OATP1B3-mediated Uptake

Yoshihisa SHITARA1,†, Kumiko TAKEUCHI1, Yoshiko NAGAMATSU1, Satomi WADA1, Yuichi SUGIYAMA2 and Toshiharu HORIE1,*

1Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan
2Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Cyclosporin A (CsA) causes a number of clinically relevant drug–drug interactions (DDIs) by inhibiting OATP1B1 and OATP1B3. In the present study, long-lasting inhibitory effects of CsA on these transporters were examined in comparison to tacrolimus (TCR). OATP1B1- and OATP1B3-expressing HEK293T cells, OATP1B1-expressing MDCK II cells, and human hepatocytes were preincubated with CsA or TCR, and uptake studies were carried out in their presence or absence. Western blot or immunohistochemical studies were done in OATP1B1-expressing HEK293T cells. The pretreatment of OATP1B1- and OATP1B3-expressing cells with 0.5–10 µM CsA, but not TCR, resulted in a reduction in their activity, even after washing out CsA from the incubation media. Preincubating the cells with CsA significantly enhanced its inhibitory effects on OATP1B1 and OATP1B3 by coincubation at 0.1–1 µM. Preincubation with 1 µM CsA caused a reduction in OATP1B1 activity for at least 18 h after its removal. The expression of OATP1B1 was not affected by incubation with CsA and no obvious change in its intracellular localization was observed. The long-lasting inhibition by CsA was also observed in human hepatocytes. Thus, CsA has a long-lasting inhibitory effect on OATP1B1 and OATP1B3. It may attribute to the clinically relevant DDIs between OATP substrates and CsA.

Keywords: OATP1B1; OATP1B3; long-lasting inhibition; drug–drug interaction; cyclosporin A

Introduction

Organic anion transporting polypeptide 1B1 (OATP1B1) and OATP1B3 are exclusively localized on the sinusoidal membranes of human hepatocytes under normal conditions although OATP1B3 was reported to also be expressed in tumor tissues.1–7 As they have broad substrate specificity, they play an important role in the hepatic uptake of many endogenous substrates and xenobiotics, including a number of therapeutic reagents.6,8 The liver is one of the most important tissues for drug elimination from the body and, thus, transporter-mediated hepatic uptake can be a determinant of the elimination rates of many drugs.9,10 In fact, many clinical studies in subjects carrying genetic polymorphisms in OATP1B1 have shown the importance of this transporter in the dispositions of many drugs in clinical situations.11–14 Recently, the International Transporter Consortium produced a list of important transporters that should be subjected to pharmacokinetic studies, which include OATP1B1 and OATP1B3.15

Recently, pharmacotherapy using multiple drugs has become common. As OATP1B1 and OATP1B3 have broad substrate specificity, concomitantly administered drugs sometimes inhibit the hepatic uptake of other drugs mediated by these transporters, resulting in drug–drug interactions (DDIs).10,13,14 Hepatic uptake transporters play a crucial role as a determinant of drug elimination in the liver for substrates of these transporters, even when they are
metabolized after transporter-mediated hepatic uptake.\textsuperscript{9,10} We have previously shown that cyclosporin A (CsA) inhibits OATP1B1, which could be the mechanism responsible for the clinically-reported DDIs between cerivastatin and CsA.\textsuperscript{16,17} Since the above study was published, many examples of clinically relevant DDIs caused by the inhibition of OATPs have been reported, and there has been an increasing focus on transporter-mediated DDIs.\textsuperscript{18,19}

Among the OATP inhibitors, CsA causes a number of clinically relevant DDIs, which cause marked pharmacokinetic alterations.\textsuperscript{10,14,15} On the other hand, tacrolimus (TCR), another widely used immunosuppressant, does not cause OATP-mediated DDIs.\textsuperscript{20,21} Hirano et al. (2006) investigated the inhibitory effects of OATP1B1 inhibitors and showed that CsA is a potent inhibitor that causes OATP1B1-mediated DDIs at therapeutic concentrations while TCR cannot.\textsuperscript{22} However, it should be noted that CsA increases the plasma concentrations of other drugs to a higher extent than is predicted by the concentration to kinetic alterations.\textsuperscript{10,14,15}

On the other hand, CsA increases the plasma concentrations of other drugs to a higher extent than is predicted by the concentration to kinetic alterations.\textsuperscript{10,14,15} The inhibitory effect of CsA on the hepatic uptake of sulfobromophthalein (BSP) in rats in vivo and its uptake in isolated rat hepatocytes, and showed that CsA has a long-lasting inhibitory effect on the hepatic uptake of BSP, which may be mediated by Oatp1a1 and Oatp1b2.\textsuperscript{23} More recently, Amundsen et al. (2010) reported that preincubation with CsA enhanced its inhibitory effect on the OATP1B1-mediated uptake of atorvastatin.\textsuperscript{24} In the present study, we investigated the inhibitory effect of CsA on human OATP1B1 and OATP1B3, focusing on long-lasting inhibition, and compared them to the inhibitory effect of TCR.

**Materials and Methods**

**Reagents:** \[^3^H\]Estrone 3-sulfate (E,S) (57.3 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). \[^3^H\]Cholecystokinin octapeptide (CCK8) (93.0 Ci/mmol) and \[^3^H\]CsA (7 Ci/mmol) were purchased from GE Healthcare (Buckinghamshire, UK). E,S was purchased from Sigma-Aldrich (St. Louis, MO). CCK8 was purchased from Peptide Institute, Inc. (Osaka, Japan). CsA was purchased from Wako Pure Chemicals (Osaka, Japan). TCR was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). 3-[4,5-Dimethylthiazol-2-yI]-2,5-di-phenyl tetrazolium bromide (MTT) was purchased from Acros Organics (Geel, Belgium). All other reagents were of analytical grade.

**Cell culture:** The methods used to produce the stably transfected HEK293T cells expressing OATP1B1 or OATP1B3, and the vector (pcDNA3.1(+))-transfected cells were described previously.\textsuperscript{25} Transporter-expressing or vector-transfected HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, low glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B at 37°C under 5% CO\textsubscript{2}. The construction of stably transfected MDCK II cells expressing OATP1B1 was described previously.\textsuperscript{26} OATP1B1-expressing MDCK II cells were grown in DMEM (high glucose) supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B at 37°C under 5% CO\textsubscript{2}.

**MTT assay:** Transporter-expressing or vector-transfected HEK293T cells were seeded in 96-well plates (Nunc, Roskilde, Denmark) coated with poly-L-ornithine/poly-L-lysine (Sigma-Aldrich) at a density of 1.2 × 10\textsuperscript{4} cells/well. The cells were cultured in the culture medium described above and, after 3 days, the cell culture medium was replaced with that containing 10 mM sodium butyrate (Wako Pure Chemicals), before being cultured for 1 more day. Then, the cells were washed with serum-free DMEM and cultured in 0.1 mL serum-free DMEM containing 0–10 µM CsA for 60 min. Subsequently, CsA-containing DMEM was removed and cells were cultured in 0.1 mL culture medium containing 0.5 mg/mL MTT for 4 h. Then, the resulting formazan was dissolved by addition of 0.1 mL well isopropylalcohol acidified with 0.4 N HCl and incubated at 37°C overnight. The absorbance at 570 nm was read using a microplate reader (Multiskan JX, Thermo Fisher Scientific, Waltham, MA).

**Uptake studies using OATP1B1- and OATP1B3-expressing HEK293T cells or OATP1B1-expressing MDCK II cells:** Prior to the uptake studies, transporter-expressing or vector-transfected HEK293T cells were seeded in 12-well plates (BD Biosciences, Franklin Lakes, NJ) coated with poly-L-ornithine/poly-L-lysine (Sigma-Aldrich) at a density of 1.5 × 10\textsuperscript{5} cells/well, and OATP1B1-expressing MDCK II cells were seeded in 24-well cell culture inserts (BD Biosciences) at a density of 1.4 × 10\textsuperscript{5} cells/well. The cells were cultured in the culture medium described above. After 3 days, the cell culture medium was replaced with that containing 10 mM sodium butyrate (Wako Pure Chemicals) for the induction of transporter expression, before being cultured for 1 more day. Before the uptake study, the cells were washed twice with ice-cold Krebs Henseleit buffer (KHB). Then, the ice-cold KHB was replaced with KHB at 37°C and prewarmed for 10 min. For the HEK293T cells, uptake was initiated by replacing the KHB with that containing \[^3^H\]E,S, \[^3^H\]CCK8, or \[^3^H\]CsA (45.0 µCi/L), and unlabeled compounds were used to adjust the final concentration. For the MDCK II cells, the KHB on the basal side was replaced with that containing \[^3^H\]E,S (225 µCi/L) to start the uptake reaction, and unlabeled E,S was used to adjust the final concentration. The reaction was terminated by removing the substrate solution by suction, and the cells were washed 4 times with ice-cold KHB. To examine the effect of pretreatment with CsA or
TCR, transporter-expressing cells were exposed to 0–10 µM of the relevant compound in serum-free media for a designated period, and uptake studies were conducted in their absence. For the inhibition studies, transporter-expressing cells were exposed to 0–1 µM CsA or TCR in serum-free medium for 0, 20, or 60 min and the uptake studies were performed in the presence of 0–1 µM of the same compound. To examine the long-lasting inhibitory effect of CsA, transporter-expressing cells were exposed to 0 or 1 µM CsA in serum-free medium for 30 min, and washed with the culture medium before being cultured in normal culture medium for a designated period; the uptake studies were conducted in the absence of CsA. The cells were dissolved in 0.5 mL of 0.1 N NaOH overnight, before being neutralized with 0.5 mL of 0.1 N HCl. Then, 800 µL aliquots and 100 µL of the incubation buffer were transferred to scintillation vials, and the radioactivity associated with the cells and that in the incubation buffer were assessed (LSC-6100, Aloka, Tokyo, Japan). Fifty microliters of cell lysate was used for the protein assay by the Lowry method using bovine serum albumin (BSA) as a standard.25

Uptake studies using primary culture of human hepatocytes: Cryopreserved human hepatocytes (59-year-old African American female, Celsis IVT, Baltimore, MD) were thawed at 37 °C, suspended in thawing medium without glucose (Biopredic International, Rennes, France), and centrifuged at 160 × g for 2 min. Hepatocytes were resuspended in William’s medium E supplemented with 10% FBS, 4 µg/mL bovine insulin, 100 µg/mL penicillin, and 100 µg/mL streptomycin and cultured in a collagen-coated 24-well plate (BD Biosciences) at a density of 3.75 × 10⁵ cells/well. After 4 h, the culture medium was replaced with serum-free William’s medium E supplemented with 10% FBS, 4 µg/mL bovine insulin, 100 IU/mL penicillin, 100 µg/mL streptomycin and 50 µM hydrocortisone hemisuccinate, and cultured for 30 min in the presence or absence of 1 µM CsA, before being used in the uptake study. Prior to the uptake studies, hepatocytes were washed twice with ice-cold KHB. Then, the ice-cold KHB was replaced with KHB at 37 °C and prewarmed for 10 min. Uptake was initiated by replacing the KHB with that containing [³H]E₁S (45.0 µCi/L), and unlabeled E₁S was used to adjust the final concentration. For the inhibition studies, CsA was added to the incubation buffer. The reaction was terminated by removing the substrate solution by suction and washing the cells 4 times with ice-cold KHB. Then, the hepatocytes were dissolved in 0.5 mL of 0.1 N NaOH overnight, before being neutralized with 0.5 mL of 0.1 N HCl. Then, 800 µL aliquots and 100 µL of the incubation buffer were transferred to scintillation vials, and the radioactivity associated with the cells and that in the incubation buffer were assessed (Tri-Carb 2300 TR, ParkinElmer Inc., Waltham, MA). Twenty microliters of cell lysate was used for the protein assay by the Lowry method using BSA as a standard.27

Western blot analysis: Transporter-expressing or vector-transfected cells were seeded in 6-well plates coated with poly-L-ornithine/poly-L-lysine at a density of 4.0 × 10⁵ cells/well and cultured in the culture medium. After 3 days, the culture medium was replaced with that containing 10 mM sodium butyrate, and the cells were cultured for 1 more day. The cells were then washed 4 times with ice cold phosphate buffered saline (PBS) and lysed in 25 mM HEPES-Tris buffer (pH 7.4) containing 10% glycerol, 1% Triton X-100, 5 µg/mL leupeptin, 1 µg/mL pepstatin, 5 µg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate (5 µg protein) was diluted with an equal volume of 2× sample buffer (0.1 M Tris-HCl (pH 6.8) containing 1% SDS, 12% mercaptoethanol, 16% glycerol, and 0.001% bromophenol blue) and separated on 8.5% SDS-polyacrylamide gel with a 3.75% stacking gel at 20 mA. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immunobilon-P transfer membrane filter; Millipore Corporation, Bedford, MA) at 15 V for 1 h. The membrane was blocked with Tris buffered saline containing 0.05% Tween 20 (TBS-T) and 3% BSA for 1 h at room temperature, before being incubated in TBS-T containing 3% BSA and 500-fold diluted anti OATP1B1 rabbit antisera overnight at 4°C. For the band detection, the membrane was incubated in TBS-T containing 3% BSA and 5,000-fold diluted horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA), and enzyme activity was assessed using the ECL plus Western blotting detection system (GE Healthcare) and a luminescent image analyzer (ChemiDoc XRS Plus, Bio-Rad Laboratories, Inc., Hercules, CA). The molecular weight was determined using a prestained protein marker (New England Biolabs, Ipswich, MA).

Immunohistochemical analysis of OATP1B1: OATP1B1-expressing or vector-transfected HEK293T cells were grown on coverslips coated with poly-L-ornithine/poly-L-lysine (Sigma-Aldrich) in 12-well plates (BD Biosciences) at a density of 1.5 × 10⁴ cells/well. After 3 days, the cell culture medium was replaced with that containing 10 mM sodium butyrate (Wako Pure Chemicals) for the induction of transporter expression, before being cultured for 1 more day. The cells were pretreated with or without 1 µM CsA for 30 min and washed twice with PBS, before being fixed for 15 min with 4% paraformaldehyde in PBS, permeabilized with 1% Triton X-100 in PBS, and blocked with 1% BSA in PBS for 1 h. Immunostaining was carried out using anti-OATP1B1 rabbit antisera (1:20) for 1 h at room temperature. Then, fluorescein anti-rabbit IgG (1:250, Invitrogen, Carlsbad, CA) in PBS containing 1% BSA was used as a secondary antibody. To stain F-actin, rhodamine phalloidin (1:25, Invitrogen) was added to the secondary antibody solution. The cells were washed 3 times with PBS and mounted onto glass slides with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). After the staining, imaging was performed using a Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss).
GmbH, Jena, Germany) equipped with argon-ion and helium-neon lasers.

Data analysis: The uptake of E1S, CCK8, and CsA into the transporter-expressing cells is expressed as the uptake volume [µL/mg protein] and determined from the radioactivity taken up into cells [dpm/mg protein] divided by that in the incubation buffer [dpm/µL]. The kinetic parameters for the uptake of E1S into the OATP1B1-expressing cells were estimated using the following equation:

\[
v_0 = \frac{V_{\text{max}} \cdot S}{K_m + S} + P_{\text{dif}} \cdot S
\]

where \(v_0\) is the initial uptake rate [pmol/min/mg protein] of E1S, as estimated from its uptake over 2 min; \(S\) is the initial concentration of E1S [µM]; and \(K_m\), \(V_{\text{max}}\), and \(P_{\text{dif}}\) are the Michaelis constant [µM], maximum uptake rate [pmol/min/mg protein], and nonsaturable uptake clearance [µL/min/mg protein] for the uptake of E1S, respectively. Fitting analyses were performed using SAAM II (University of Washington, Seattle, WA). Statistical comparisons among multiple groups were conducted using ANOVA followed by Dunnett’s test. Difference was considered to be significant when \(p < 0.05\).

Results

Effect of CsA pretreatment on OATP1B1- and OATP1B3-mediated transport: The uptake of 0.01 µM of \([3H]E1S\) and \([3H]CCK8\) in OATP1B1- and OATP1B3-expressing HEK293T cells, respectively, was examined in the absence of CsA or TCR after pretreatment with the same compounds for a designated period. CsA and TCR significantly inhibited the OATP1B1- and OATP1B3-mediated uptake in cells that had not been pretreated with the

Fig. 1. OATP1B1- or OATP1B3-mediated uptake after preincubation with CsA or TCR
The uptake of \([3H]E1S\) (a, c) and \([3H]CCK8\) (b, d) in OATP1B1- and OATP1B3-expressing HEK293T cells was examined in the absence of CsA (a, b) or TCR (c, d) after preincubation with the same compound for a designated period. Data are shown as percentage mean uptake clearance values ± S.E. \((n = 6)\) compared with those of the control (preincubation for 5 min in CsA- or TCR-free media). **\(p < 0.01\) vs. incubation without CsA; *\(p < 0.05\), **\(p < 0.01\) vs. 5 min incubation.
same compounds. The IC₅₀ values of CsA on OATP1B1 and OATP1B3 were within 0.1–1 µM while those values for TCR were higher than 1 µM. CsA and TCR inhibited OATP1B1- and OATP1B3-mediated uptake also in cells that had been subjected to pretreatment. CsA pretreatment enhanced its inhibitory effects on OATP1B1 and OATP1B3 at 0.1 and 1 µM while TCR pretreatment did not enhance its inhibitory ability at any concentration.

Long-lasting inhibition of OATP1B1- and OATP1B3-mediated uptake by CsA: After preincubation with 1 µM CsA for 30 min, the transporter-expressing cells were washed with culture media and cultured for 0–18 h in normal culture media without CsA, and the uptake of 0.01 µM [³H]E₁S and [³H]CCK8 in the OATP1B1- and OATP1B3-expressing HEK293T cells was examined in the absence of CsA (Fig. 3). In the OATP1B1-expressing cells, transporter-mediated uptake was significantly lower than that in the CsA-untreated control cells even at 18 h after the preincubation step, although no significant long-lasting inhibitory effect was observed in the OATP1B3-expressing cells. Just after preincubation with 1 µM CsA, the OATP1B1-mediated uptake was 8.55 ± 0.51% of that seen in the CsA-untreated controls. Incubation in CsA-free medium recovered the OATP1B1-mediated uptake in a culture time-dependent manner, and it was 48.4 ± 2.0% of the value in the control (p < 0.01 vs. CsA-free control) after 18 h incubation.

Kinetic analysis of the uptake of E₁S in the OATP1B1-expressing cells pretreated with or without CsA: The kinetic parameters for the uptake of [³H]E₁S (0.01–100 µM) were determined in OATP1B1-expressing HEK293T cells that had been pretreated with or without 1 µM CsA. Figure 4 shows Eadie–Hofstee plots for the uptake of [³H]E₁S in these cells. Kinetic analyses revealed the Kₘ, Vₘₐₓ, and Pₐₙ to be 0.0593 ± 0.0225 µM, 4.11 ± 0.89 pmol/min/mg protein, and 1.31 ± 0.21 µL/min/mg protein, respectively, for the CsA-untreated OATP1B1-expressing cells in the absence of CsA. The corresponding values for the CsA-pretreated OATP1B1-expressing cells were 0.376 ± 0.281 µM, 2.69 ± 1.53 pmol/min/mg protein, and 0.565 ± 0.215 µL/min/mg protein, respectively. The same studies were performed in the presence of 1 µM CsA. The Kₘ, Vₘₐₓ, and Pₐₙ obtained in the presence of 1 µM CsA in the CsA-untreated OATP1B1-expressing cells were 0.250 ±
The uptake of \(^{[3]H}\)E1S in the OATP1B1-expressing cells was examined in the absence of CsA after preincubation with \(^{[3]H}\)E1S (0.01 \(\mu \text{M}\)) was examined (Fig. 7). The OATP1B1-mediated uptake of \(^{[3]H}\)E1S was significantly reduced by pretreatment with 1 \(\mu \text{M}\) CsA on the basal side. It was also significantly reduced by pretreatment on the apical side of the cells to a level similar to that produced by pretreatment on the basal side. The concentration of CsA in the medium on the basal side of the cells (OATP1B1-expressing side) and the amount taken up into the cells were examined after \(^{[3]H}\)CsA (1 \(\mu \text{M}\)) was added to the apical or basal side of the cells in serum-free culture medium and incubated for 1 h at 37°C (Fig. 8). When \(^{[3]H}\)CsA was added to the apical side, it was minimally transported to the basal side, and the concentration of CsA was 0.0126 ± 0.0008 \(\mu \text{M}\) after 1 h incubation. When it was added to the basal side, its concentration on the basal side was 0.842 ± 0.041 \(\mu \text{M}\) after 1 h incubation. During the 1 h incubation period, CsA was taken up into the cells in an incubation time-dependent manner. The amounts of CsA taken up into the cells from the basal and apical sides were comparable.

### Effect of pretreatment with CsA on the uptake of \(^{[3]H}\)E1S in primary culture of human hepatocytes

The effect of CsA pretreatment on the uptake of \(^{[3]H}\)E1S (0.1 \(\mu \text{M}\)) was examined in primary culture of human hepatocytes (Fig. 9). After pretreatment with 1 \(\mu \text{M}\) CsA for 30 min, the uptake of \(^{[3]H}\)E1S was examined in the absence of CsA. \(^{[3]H}\)E1S uptake was significantly reduced to 62.1 ± 8.2% of that observed in the control cells. The addition of 1 \(\mu \text{M}\) CsA also reduced the uptake of \(^{[3]H}\)E1S to 77.7 ± 5.1% of that in the control although the effect was not significant.

### Discussion

Previously, we have shown that CsA has a long-lasting inhibitory effect on the uptake of BSP in rat hepatocytes, which is presumably mediated by Oatp1a1 and Oatp1b2.\(^{23}\) In the present study, we comparatively examined the inhibitory effects of CsA and TCR on human OATP1B1 and OATP1B3, focusing on their long-lasting inhibition. Pretreatment with CsA at concentrations higher than 0.5 \(\mu \text{M}\) caused a significant reduction in OATP1B1 and OATP1B3 activity, even after its removal (Fig. 1), which
is similar to its effect on the uptake of BSP in primary cultured rat hepatocytes. On the other hand, TCR did not have inhibitory effects on OATP1B1 or OATP1B3 after its removal, although its presence inhibited both transporters (Figs. 1 and 2). Thus, CsA is a long-lasting inhibitor of OATP1B1 and OATP1B3 while TCR is a normal inhibitor.
of these transporters. In the present study, we showed that the inhibitory effects by 0.5 and 1 µM CsA on OATP1B1 and OATP1B3 were incubation time-dependent, whereas this was not the case in our previous study using rat hepatocytes.

Preincubation with CsA enhanced its inhibitory effect on OATP1B1 and OATP1B3, but this was not the case for TCR. A similar result was reported by Amundsen et al. (2010) for their inhibition of the OATP1B1-mediated uptake of atorvastatin. The mechanism can be explained by the long-lasting inhibition induced by preincubation with CsA. The IC50 values for the effects of CsA and TCR on OATP1B1-mediated uptake were reported to be 0.2–0.5 and 0.6–3.7 µM, respectively, which were consistent with the values obtained by coincubation with CsA and TCR in the present study.17,22,24,28

The inhibitory effect of CsA on human hepatic uptake transporters continues for hours after its removal in in vitro studies, although we have previously shown that its inhibitory effect was observed for at least 3 days in rats in vivo.23 Thus, CsA may potently inhibit the hepatic uptake of drugs mediated by OATP1B1 and OATP1B3 in clinical situations even after it has been eliminated from the body. This may partially explain the mechanism responsible for the
clinically reported DDIs with CsA caused by the inhibition of OATP1B1. Preincubation with CsA markedly changed the $K_m$ value, rather than $V_{\text{max}}$, for the OATP1B1-mediated uptake of E1S, which was similar with the case for the long-lasting inhibition of the uptake of BSP in rat hepatocytes by incubation with CsA. $^{23}$ Coincubation with CsA also changed the $K_m$ value rather than $V_{\text{max}}$, suggesting it inhibits OATP1B1 in a competitive manner. In the present study, coincubation after preincubation with CsA more markedly changed the $K_m$ value with a reduction of $V_{\text{max}}$ value comparing to the control study (uptake study in the absence of CsA in untreated cells). Thus, pretreatment of OATP1B1-expressing cells with CsA mainly changed the affinity for E1S rather than the capacity. In fact, the long-lasting reduction in OATP1B1 activity observed after CsA incubation was not associated with reduced expression of this transporter (Fig. 5). In the present study, we compared the expression level of this transporter in total cell lysate prepared from CsA-treated and -untreated cells because most transporters are reportedly detected on the cell surfaces of OATP1B1-expressing HEK293 cells. $^{4,29}$ However, the internalization of OATP2B1 from the cell surface was recently reported. $^{30}$ By this mechanism, the activity of this transporter could be reduced without changes in its total expression level. Thus, we examined the intracellular localization of OATP1B1 in CsA-treated cells by immunohistochemical analyses but we could not show an obvious alteration in its intracellular localization (Fig. 5). It may suggest that altered localization of OATP1B1 was not associated with the reduced activity of this transporter by preincubation with CsA. But the present result did not strongly support that because the immunostained image of OATP1B1 was not clear, possibly due to non-specific binding of the antibody. Köck et al. (2010) showed that the total expression of OATP2B1 was markedly reduced at 4h after its internalization because of the induction of a subsequent degradation pathway. $^{30}$ On the other hand, in the present study, the expression level of OATP1B1 was not altered even after 4h-incubation with 1 $\mu$M CsA (data not shown).

In our previous study using rats, CsA and its metabolites were detected in the liver, even after it had mostly disappeared from the systemic circulation. Thus, CsA and/or its metabolites in the liver may affect transporter function. Thus, the intracellular level of CsA was examined in transporter-expressing and vector-transfected cells after its removal from the incubation buffer (Fig. 6). The OATP1B1- and OATP1B3-mediated uptake of CsA was not examined in the present study. This suggests that CsA is not a substrate of these transporters although it is an inhibitor, or that transporter-mediated uptake was too low to detect due to extensive binding and/or passive diffusion in the cells. After the removal of CsA from the incubation buffer and further incubation in CsA-free medium, its amount in the cells was significantly decreased, but more than 50% remained in the cells for 18h. Thus, CsA in the cells or on the cell surface may affect transporter activity.

To determine whether cell surface-associated CsA on the OATP1B1-expressing side or that inside the cells caused the long-lasting inhibition of OATP1B1, we used OATP1B1-expressing MDCK II cells. As OATP1B1 is exclusively localized on the basal side of these cells, $^{26}$ the effects of adding CsA to the basal or apical side were compared. OATP1B1 was significantly inhibited even when CsA was added to the apical side, where OATP1B1 is not localized, and then removed (Fig. 7). When 1 $\mu$M CsA was added to the apical side, its concentration on the basal side was not sufficient for either competitive or long-lasting OATP1B1 inhibition (Figs. 1 and 8). These observations suggest that the exposure of the substrate binding site of OATP1B1 to CsA is not required for the long-lasting inhibitory effects of CsA, but rather they are caused by the CsA inside the cells or that exposed from within the cells. The intracellular level of CsA was comparable regardless of whether CsA was added to the basal or apical side prior to 1h incubation (Fig. 8). Considering that CsA inhibits OATP1B1 from outside as well as inside of the cells, enhancement of OATP1B1 inhibition by preincubation + coincubation can be explained (Figs. 2 and 4).

In the present study, CsA was shown to be a long-lasting inhibitor of OATP1B1 and OATP1B3. This effect was also demonstrated in human hepatocytes (Fig. 9), suggesting that it may occur for humans in clinical situations. In the case of metabolic enzymes, long-lasting inhibitors cause serious interactions. $^{31-33}$ Thus, the mechanism underlying the severe DDI of CsA mediated by OATP inhibition may be explained by the long-lasting inhibition described in the current study. On the other hand, TCR causes a lower number of clinically relevant DDIs because it minimally inhibits OATP1B1 at therapeutic concentrations $^{32}$ and, in addition, it does not have a long-lasting inhibitory effect.
To avoid remarkable DDIs caused by long-lasting inhibition, the mechanisms of transporter inhibition should be studied during drug discovery and development. During the clinical use of CsA with OATP1B1 or OATP1B3 substrates, we should be careful to avoid adverse reactions caused by more marked DDIs than expected.

Acknowledgments: We are grateful for Astellas Pharma Inc. for kindly providing the tacrolimus. We are also grateful for Drs. Ken-ichi Kaneko, Mikako Torii, Tomoko Yamaguchi, Takefumi Nakano and Hiroshi Iwata in Mitsubishi Chemical Medience Corporation for technical support with human hepatocyte primary culture.

References


12) Ieiri, I., Higuchi, S. and Sugiyama, Y.: Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for inter-individual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. *Expert Opin. Drug Metab. Toxicol.*, 5: 703–729 (2009).


27) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.:


