Evaluation of Oatp and Mrp2 Activities in Hepatobiliary Excretion Using Newly Developed Positron Emission Tomography Tracer \[^{11}C\text{]Dehydropravastatin in Rats\textsuperscript{5}}\]

Tomotaka Shingaki, Tadayuki Takashima, Ryosuke Ijiuin, Xuan Zhang, Tomohiro Onoue, Yumiko Katayama, Takashi Okauchi, Emi Hayashinaka, Yilong Cui, Yasuhiro Wada, Masaaki Suzuki, Kazuya Maeda, Hiroyuki Kusuhara, Yuichi Sugiyama, and Yasuyoshi Watanabe

RIKEN Center for Life Science Technologies (T.S., Y.K., T.Ok., E.H., Y.C., Y.Wad., Y.Wat.) and RIKEN Center for Molecular Imaging Sciences, Kobe, Hyogo, Japan (T.S., T.T., R.I., T.Ok., Y.K., E.H., Y.C., Y.Wad., M.S., Y.Wat.); Laboratory of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan (X.Z., T.On., K.M., H.K.); and Sugiyama Laboratory, RIKEN Innovation Center, Yokohama Bio Industry Center, Yokohama, Kanagawa, Japan (Y.S.)

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ABSTRACT

We developed a pravastatin derivative, sodium (3R,5R)-3,5-dihydroxy-7-((1S,2S,6S,8S)-6-hydroxy-2-methyl-8-((1-[\(^{11}\)C]-E)-2-methyl-but-2-enoyl)oxy)-1,2,6,7,8,8a-hexahydropyridazin-1-yl)heptanoate ([\(^{11}\)C]DPV), as a positron emission tomography (PET) probe for noninvasive measurement of hepatobiliary transport, and conducted pharmacokinetic analysis in rats as a feasibility study for future clinical study. Transport activities of DPV in freshly isolated rat hepatocytes and rodent multidrug resistance–associated protein 2 (rMrp2; human, MRP2)-expressing membrane vesicles were similar to those of pravastatin. Rifampicin diminished the uptake of DPV and pravastatin by the hepatocytes, with similar inhibition potency. [\(^{11}\)C]DPV underwent biotransformation to produce at least two metabolites in rat, but metabolism of [\(^{11}\)C]DPV occurred negligibly in human hepatocytes during a 90-minute incubation. After intravenous injection, [\(^{11}\)C]DPV was mainly distributed to the liver and kidneys, where the tissue uptake clearances (CL\textsubscript{uptake,liver} and CL\textsubscript{uptake,kidney}) were blood-flow–limited (73.6 ± 4.8 and 24.6 ± 0.6 ml/min per kilogram, respectively). Systemic elimination of [\(^{11}\)C]DPV was delayed in rifampicin-treated rat and an Mrp2-deficient mutant rat, Eisai hyperbilirubinic mutant rat (EHBR). Rifampicin treatment decreased both CL\textsubscript{uptake,liver} and CL\textsubscript{uptake,kidney} of [\(^{11}\)C]DPV by 30% (P < 0.05), whereas these parameters were unchanged in EHBR. Meanwhile, the canalicul efflux clearance (CL\textsubscript{eff,canal}) of [\(^{11}\)C]DPV, which was 12.2 ± 1.5 ml/min per kilogram in the control rat, decreased by 60% and 89% in rifampicin-treated rat and EHBR (P < 0.05), respectively. These results indicate that [\(^{11}\)C]DPV is taken up into the liver by organic anion-transporting polypeptides (rodent, Oatps; human, OATPs) and multidrug resistance–associated protein 2 (rodent, Mrp2; human, MRp2) are believed to mediate the hepatobiliary transport of a variety of drugs, especially those with anionic properties, and endogenous conjugated metabolites (Nies et al., 2008; Yoshida et al., 2013). Although clinical concerns were described in a white paper published by the International Transporter Consortium (Giacomini et al., 2010), and draft guidelines/guidelines were issued by the Food and Drug Administration and European Medicines Agency, the concerted manner of action of OATPs and MRp2 based on clinical observation has not been fully discussed yet because of limitations in the direct and separate measurement of influx and canalicular efflux clearances by conventional clinical studies using nonradioabeled drugs.

Introduction

It is well accepted that drug transporters play important roles in drug disposition, on the basis of the results from pharmacogenomics and drug-drug interaction (DDI) studies (Giacomini et al., 2010). Multispecific transporters such as organic anion-transporting polypeptides (rodents, Oatps; human, OATPs) and multidrug resistance–associated protein 2 (rodents, Mrp2; human, MRp2) are believed to mediate the hepatobiliary transport of a variety of drugs, especially those with anionic properties, and endogenous conjugated metabolites (Nies et al., 2008; Yoshida et al., 2013). Although clinical concerns were described in a white paper published by the International Transporter Consortium (Giacomini et al., 2010), and draft guidelines/guidelines were issued by the Food and Drug Administration and European Medicines Agency, the concerted manner of action of OATPs and MRp2 based on clinical observation has not been fully discussed yet because of limitations in the direct and separate measurement of influx and canalicular efflux clearances by conventional clinical studies using nonradioabeled drugs.

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There are high expectations that quantitative pharmacokinetic analysis involving tissue concentrations will provide insight into the mechanisms of drug distribution and clearance, as well as DDIs for drug development. Positron emission tomography (PET) studies enable the noninvasive analysis of drug concentrations in tissues with high sensitivity and good spatiotemporal resolution, allowing direct measurement of the intratissue distribution of drugs (Kusuhara, 2013). We have demonstrated the usefulness of PET molecular imaging for pharmacokinetic analysis of transporter substrates in vivo (Takashima et al., 2011a,b, 2012). For instance, $^{11}$C-labeled (15R)-16-m-toly-17,18,19,20-tetranorisorcabacryn methyl ester (15R-$^{11}$C|TIC-Me) was rapidly converted to 15R-$^{11}$C|TIC in the plasma, predominantly accumulated in the liver and kidneys, and then most of the radioactivity was excreted into bile and finally to the intestine. The hepatobiliary transport of 15R-$^{11}$C|TIC was shown to involve OATPs and MRp2. We found that, in addition to the hepatic uptake process, the canalicular efflux of radioactivity was also decreased by rifampicin in humans. However, the metabolism of 15R-$^{11}$C|TIC in both rodent and human liver may raise some difficulties in the precise evaluation of hepatobiliary and renal clearance in vivo.

For these reasons, nonmetabolizable PET probes with specificities against OATP and MRp2 are urgently required for clinical PET research. We have developed a pravastatin derivative, (3R,5R)-3,5-dihydroxy-7-(1S,2S,6S,8S)-6-hydroxy-2-methyl-8-(1-[11]Cl)|E|-2-methybut-2-en-3-ol)oxy)-1,2,6,7,8,8a-hexahydropaphthalen-1-yl|heptanoate ([$^{11}$C|DPV; Fig. 1), as a PET probe for hepatobiliary transport (Ijuin et al., 2012). We conducted qualitative investigations in rat using this probe. The PET images showed that the radioactivity after intravenous administration of [$^{11}$C|DPV predominantly accumulated in the liver and kidneys and was then excreted into the intestine via bile and the urinary bladder via urine. Moreover, the hepatobiliary excretion was inhibited by rifampicin, and was also reduced in Mrp2-deficient mutant rats (Eisai hyperbilirubinemic rats (EHBR)). These results showed that [$^{11}$C|DPV has great potential as a PET probe, enabling more detailed analysis of the excretion mechanism mediated by OATPs and MRp2 in humans. However, quantitative pharmacokinetic analysis evaluating the activities of these transporters is required for translation into clinical investigations.

In this study, taking into account the clinical investigation by PET with [$^{11}$C|DPV as a nonmetabolizable PET probe, precise investigations were carried out to obtain insight into hepatobiliary transport involving Oatps and Mrp2 with control and rifampicin-treated rats, as well as with EHBR. Radiometabolite analysis was also performed in rats, and the result was compared with that obtained from cryopreserved human hepatocytes. In addition, to support the membrane transport by Oatps and Mrp2 in the liver, in vitro uptake of DPV by rat hepatocytes and rMrp2-expressing membrane vesicles was also carried out.

Materials and Methods

For in vitro transporter assessment, rat Mrp2-expressing membrane vesicles were purchased from Genombrane (Kanagawa, Japan). [$^{3}$H]Estradiol-17β-glucuronide (E$_2$17G, 40 Ci/mmol, >97%) was purchased from PerkinElmer (Waltham, MA).

For in vivo PET study, [$^{11}$C]DPV was synthesized according to the procedure reported previously (Ijuin et al., 2012). The identifications and concentration of [$^{11}$C]DPV were assessed by high-performance liquid chromatography (HPLC). Radiochemical purity and chemical purity were greater than 99 and 98%, respectively, and the specific radioactivity was 19–42 GBq/µmol at the time of injection. The purified fraction was evaporated and reconstituted with approximately 4 ml of saline. Rifampicin was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved at 22.5 mM in saline by addition of an appropriate amount of 1 M NaOH for solubilization.

Animals. For the in vitro transporter assessment, male Sprague-Dawley rats (7–10-weeks-old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were maintained under standard conditions with a reverse dark-light cycle and were treated humanely. Food and water were available ad libitum. The studies reported in this manuscript were conducted in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, the University of Tokyo, Tokyo, Japan).

For in vivo PET study, male Sprague-Dawley rats (6–7-weeks-old, $n = 3–4$ for each set of experiments) weighing 180–242 g and male hereditarily Mrp2-deficient rats (EHBR; 7–8-weeks-old, $n = 3$) weighing 287–327 g were purchased from Japan SLC, Inc. The animals were kept in a temperature- and light-controlled environment with standard food and tap water provided ad libitum. All experimental protocols were approved by the Ethics Committee on Animal Care and Use of the RIKEN Center for Life Science Technologies, and were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985).

In Vitro Uptake Study Using Freshly Isolated Rat Hepatocytes. Isolation of hepatocytes and an uptake study were conducted as described previously (Yamazaki et al., 1993). Isolated hepatocytes (viability more than 85%) were suspended in Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO$_3$, 4.8 mM KCl, 1.0 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl$_2$, pH 7.4) and stored on ice. Before the uptake study, the hepatocytes were preincubated at 37°C for 3 minutes, and the uptake reaction was started by adding test drugs to the hepatocyte suspension. After the designated time, the reaction was terminated by separating the cells from the medium using a centrifugal filtration technique. For this purpose, a 75-µl aliquot of incubation mixture was placed in a 0.2-ml centrifuge tube (Sarstedt, Numbrecht, Germany) containing 50 µl of 2 M NaOH for radiolabeled compounds or 50 µl of 5 M CH$_3$COONa for unlabeled compounds under a 100-µl layer of an oil mixture (density = 1.05, mixture of silicone oil and mineral oil; Sigma-Aldrich). Samples were then centrifuged for 10 seconds in a microfuge. During this process, the hepatocytes passed through the oil layer into the aqueous solution (2 M NaOH or 5 M CH$_3$COONa). The radioactivity of E$_2$17β-G-associated cell and medium specimens was measured using a liquid scintillation counter (LS6800SE, Beckman Coulter, Brea, CA). The concentrations of DPV and pravastatin were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Cells in 5 M CH$_3$COONa buffer were taken from the centrifuge tube and sonicated in a new tube to break them down. This sample was used for the measurement of drug concentrations by

![Fig. 1. Chemical structures of [$^{11}$C]DPV and pravastatin.](image-url)
LC-MS/MS as described in “Quantification of DPV and Pravastatin by LC-MS/MS” below.

In Vitro Uptake Study Using rMrp2-Expressing Membrane Vesicles. The vesicular transport was performed by a rapid filtration technique described previously (Ito et al., 1998). Transport buffer (15 μl; 50 mM 3-morpholinopropane-1-sulfonic acid-Tris, 70 mM KCl, 7.5 mM MgCl₂, 2 mM glutathione) containing each compound was preincubated at 37°C for 3 minutes, and then supplemented with 5 μl of membrane vesicle solution (5 μg of protein) to start the reaction. Chilled stop buffer (1 ml; 40 mM 3-morpholinopropane-1-sulfonic acid-Tris, 70 mM KCl) was added to terminate the reaction. The assay tubes were placed on ice until the filtration. The stopped reaction mixture was filtered through a 0.45-μm HAWP filter (Millipore, Billerica, MA) and then washed twice with 5 ml of stop buffer. For determination of the inhibitory effect of DPV or pravastatin on E₀₁⁷G₅uptake, the radioactivity retained on the filter was measured using a liquid scintillation counter (LS6800SE; Beckman Coulter). For determination of transport activity of DPV and pravastatin, the filters were dried and placed into tubes. Compounds were extracted with 400 μl of acetone and then centrifuged, and the supernatant was diluted 5-fold with water. Concentrations of DPV and pravastatin were measured using LC-MS/MS as described below.

Quantification of DPV and Pravastatin by LC-MS/MS. The concentrations of DPV and pravastatin were measured on an AB SCIEX QTRAP 5500 mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Prominence high-pressure liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) operated in the negative electrospray ionization mode. Chromatographic separation was achieved on an Inertsl ODS-3 column (50 mm × 2.1 mm internal diameter; GL Science, Tokyo, Japan) in binary gradient mode at a flow rate of 0.4 ml/min. The mobile phase comprised 0.1% formic acid-Tris, 70 mM KCl, and rat plasma were used for the assay. The plate was incubated at 57°C for 20 hours. Concentrations of rifampicin at both sides were analyzed by LC-MS/MS. Briefly, Micromass Quattro micro API (Waters, Milford, MA) equipped with the separation module (Waters 2695), operated in the electron spray ionization mode, was used for the analysis of rifampicin. The chromatographic separation was achieved using an Inertsil ODS-3 column (50 mm × 2.1 mm internal diameter; GL Science, Tokyo, Japan). Gradient elution with water containing 0.05% formic acid as mobile phase A and acetonitrile as mobile phase B was performed with a flow rate of 0.4 ml/min as follows: The concentration of mobile phase B was increased from 20 to 60% over 1 minute, and then kept constant. Rifampicin eluted at 3.1 minutes. Rifampicin was ionized by electrospray ionization and the following positive ions were detected in the multiple reaction monitoring mode: m/z 823.4 (precursor ion) to m/z 791.4 (product ion).

PET Scans. All PET scans were performed with a microPET Focus220 scanner (Siemens, Knoxville, TN) designed for laboratory animals, the procedure for which was briefly reported previously (Ijini et al., 2012). In detail, the rats were anesthetized and maintained with a mixture of 1.5% isoflurane and nitrous oxide/oxygen (7:3), and the femoral artery was cannulated with a polyethylene tube (SP-31; Natsume Seisakusho, Tokyo, Japan) for the collection of blood samples. Before emission scanning, the animals were placed at the center of the PET camera, and a 25-minute transmission scan with a rotating ⁶⁸Ge/⁶⁷Ga point source was performed for the abdomen positioning and attenuation correction. At the start of the emission scan, [¹¹C]DPV was administered as a single bolus via the tail vein at a dose of 97 ± 17 MBq/kg. The chemical amount of [¹¹C]DPV for the bolus injection was calculated as 0.88 ± 0.67 nmol/body (0.39 ± 0.30 μg/body). For the functional analysis of Oatps in terms of hepatic uptake, intravenous infusion of rifampicin, a typical Oatp inhibitor, was adopted at a rate of 1.5 μmol/min per kilogram from at least 90 minutes before the administration of [¹¹C]DPV to the end of the PET scan. An emission scan in 3D list-mode was performed for 60 minutes and sorted into 31 dynamic sinograms according to the following sequence: 12 × 10 seconds, 8 × 30 seconds, 8 × 180 seconds, 3 × 600 seconds. Arterial blood was sampled via the cannulated femoral artery 12 times within 60 minutes at the following time-points: 10, 20, 30, 40, and 50 seconds and 1, 2, 5, 10, 20, 40, and 60 minutes after the administration of [¹¹C]DPV. The volume of blood sampled for each time-point was within 120 μl, and the total blood volume sampled from one rat did not exceed 1.6 ml, approximately 10% of the total circulating blood volume. Blood radioactivity was measured with a 1470 Wizard Automatic Gamma Counter (PerkinElmer). The radioactivity in each measured sample was corrected for time decay from the point of [¹¹C]DPV administration.

Analysis of PET Imaging Data. PET images were reconstructed using Siemens MicroPET Manager 2.4.1.1 by Fourier Rebinning and standard 2D filtered back projection using Ramp filter with a cut-off at the Nyquist frequency. Regions of interest were automatically placed on the liver, kidneys, urinary bladder, and intestine using the isointensuring tool in the PMOD v3.3 program (PMOD Technologies Ltd., Zurich, Switzerland), as described previously (Takashima et al., 2011a; Ijini et al., 2012). ROIs were defined on tissue images at each frame in which these tissues could be easily identified. The defined ROIs were then manually corrected by fitting the images of each tissue slice and time frame without overlapping any of the defined ROIs. All ROIs were combined and changed to volumetric regions of interest. In this PET analysis, ROIs for the intestine were defined larger than those obtained from the images for calculating the total amount of radioactivity in the intestine in order to include the contribution of the partial volume effect from the bile excreted into the intestine. Therefore, the radioactivity in the intestine described in this study is “the radioactivity in bile.” Time-radioactivity curves for each tissue and region were constructed by normalizing decay-corrected time-radioactivity measurements to the injected dose (% dose) of [¹¹C]DPV.

Radiometabolite Analysis. Since the metabolites of [¹¹C]DPV were briefly analyzed in our previous study (Ijini et al., 2012), further quantitative metabolite assessment was performed on blood, liver, and bile samples in rat to evaluate the average values of time-metabolite composition profiles in these samples. The separation of [¹¹C]DPV and more than several metabolites in kidney and urine samples from rat was so difficult that further investigation was not carried out. The control and rifampicin-treated rats, as well as EHBR, were anesthetized with 1.5% isoflurane mixed with 2.5 l/min air. For bile sample collection, the bile duct was cannulated with PE-10 tubing (BD Biosciences) prior to administration. [¹¹C]DPV (116 ± 44 MBq/kg) was injected into the tail vein and then bile was collected over periods of 0 to 5, 5 to 10, 10 to 20, and 20 to 30 minutes after administration. Venous blood was collected at 2, 5, 20, and 30 minutes. To sample liver tissue, blood flow was terminated by transection of the abdominal aorta and vein at 5, 10, 20, and 30 minutes after administration, and the liver was quickly removed and homogenized using 20 ml of purified water. The blood, bile, and liver homogenates were deproteinized by precipitation with 2 volumes of acetonitrile and centrifuged at 14,000g for 2 minutes at 4°C. The supernatants of blood samples were applied to RP-8 thin-layer chromatography (TLC) plates (Merck Biosciences, Darmstadt, Germany). The plates were developed at room temperature with acetonitrile/water/formic acid (50:50:0.1) as a mobile phase. After
migration, the plates were dried and exposed to BAS TR2040 imaging plates (FujiFilm, Tokyo, Japan) for 20 minutes. The distribution of radioactivity on the imaging plates was determined by digital PSL autoradiography using a Fuji FLA-7000 analyzer at 50-μm resolution. The data were then analyzed using the MultiGauge image analysis program (FujiFilm) to calculate the proportion of parent [11C]DPV.

Radiometabolite analysis was also performed to confirm the radiometabolite production of [11C]DPV in the cultured human hepatocytes. Cryopreserved human hepatocytes obtained from Gibco (Life Technologies, Inc., Grand Island, NY) were thawed and prepared on the basis of a procedure published previously (Li et al., 2009). A suspension of hepatocytes (2.5 × 10^6 cells/ml in William’s E medium) obtained from Gibco (or medium without hepatocytes) was incubated with [11C]DPV (1 MBq) at 37°C in a humidified carbon dioxide (5%) chamber for a maximum of 90 minutes. Samples were taken at 1, 30, 60, and 90 minutes, and the sample preparation and TLC separation were carried out as described above.

Given the difficulty of identification of the radiometabolites in the liver and bile by TLC, HPLC investigation was also used. The supernatants of the liver and bile samples were diluted with HPLC mobile phase and then analyzed using an HPLC system (Shimadzu) with a coupled NaI(Tl) positron detector, UG-SCA30 (Universal Giken, Kanagawa, Japan) to measure the intact radiotracer and its metabolites. Chromatographic separation was achieved on a Cosmosil Hilic column (150-mm × 4.6-mm internal diameter; Nacalai Tesque, Kyoto, Japan) in binary gradient mode at a flow rate of 1.5 ml/min. The mobile phase comprised 10 mM ammonium acetate (pH 7.4) and acetonitrile. The concentration of acetonitrile was initially 90%, it was then linearly decreased to 48% over 5 minutes, and maintained for a further 4 minutes. Finally, the column was re-equilibrated with acetonitrile at a concentration of 90% over 6 minutes. The elution was monitored by UV absorbance at 254 nm and coupled NaI positron detector. The individual peak areas of the radiometric HPLC chromatograms were corrected for the radioactive decay to the retention time of radiometabolite for each sample and expressed as fractions of total radioactivity.

**Data Calculation for In Vitro Kinetic Parameters on the Uptake Study Using Freshly Isolated Rat Hepatocytes.** Kinetic parameters for the specific uptake by rat Oatp were obtained from eq. 1 (Michaelis-Menten equation):

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

where \(v\) is the uptake rate of the substrate (pmol/min per milligram of protein), \(S\) is the substrate concentration in the medium (μM), \(K_m\) is the Michaelis constant (μM), \(V_{\text{max}}\) is the maximum uptake rate (pmol/ min per milligram protein), and \(P_{\text{dif}}\) is the clearance for nonsaturable component (μl/min per milligram protein). The experimental data were fitted to the equation using nonlinear regression analysis with weighting as the reciprocal of the observed values, using the MULTI program; the damping Gauss-Newton method algorithm was used for curve fitting (Yamaoka et al., 1981).

**In Vivo Kinetic Analysis for the Clearance by PET Imaging Analysis.** Initial [11C]DPV uptake rates for the rat liver or kidneys were calculated by the integration plot method (Kim et al., 1988), using the portion of time-radioactivity curves encompassing approximately the first 1 minute of the linear range of the plot after [11C]DPV administration, during which the effects of the metabolism and excretion from the tissue were negligible. The uptake clearance of [11C]DPV was determined using eq. 2:

\[
\frac{X_{\text{tissue}}}{C_{\text{blood}}} = \frac{CL_{\text{uptake,tissue}} \cdot AUC_{0-\text{t,blood}}}{C_{\text{blood}}} + \frac{V_F \cdot X_{\text{tissue}}}{C_{\text{blood}}}
\]

where \(X_{\text{tissue}}\) is the amount of radioactivity as [11C]DPV in the liver or kidneys at time \(t\) as determined by PET image analysis, and \(C_{\text{blood}}\) is the concentration of radioactivity as [11C]DPV in the blood at time \(t\), as determined by gamma counter measurement of the radioactivity in the blood taken from the femoral artery. The tissue uptake clearance (CL\(_{\text{uptake,liver}}\) and CL\(_{\text{uptake,kidney}}\)) was obtained from the initial slope (0.3–0.9 minutes, showing the linearity) of the plot of \(X_{\text{tissue}}/C_{\text{blood}}\) versus AUC\(_{0-\text{t,blood}}\) (AUC\(_{0-\text{t,blood}}\) is the area under the blood concentration-time curve from time 0 to time \(t\). \(V_F\) is the initial distribution volume in the liver or kidneys at time 0, calculated from the y-intercept of the plot.

Canalicular efflux clearance (CL\(_{\text{ent,bile}}\)) was estimated using the following equation by noninvasive measurements of the radioactivity in the liver and intestine (the radioactivity in bile excreted into the intestine) in the periods of approximately 1–3 minutes and 6–8 minutes after drug administration:

\[
X_{\text{bile}} = CL_{\text{ent,bile}} \cdot AUC_{0-\text{t,liver}} + V_F
\]

where \(X_{\text{bile}}\) is the amount of radioactivity in the intestine (the radioactivity in bile excreted into the intestine) at time \(t\), as determined by PET image analysis. CL\(_{\text{ent,bile}}\) represents the canalicular efflux clearance of radioactivity in the liver, and AUC\(_{0-\text{t,liver}}\) is the area under the hepatic concentration-time curve from time 0 to time \(t\). The CL\(_{\text{ent,bile}}\) value can be obtained from the slope of the plot of \(X_{\text{bile}}\) versus AUC\(_{0-\text{t,liver}}\). \(V_F\) was calculated as the y-intercept of the integration plot.

**Statistical Analysis.** For the in vitro transporter assessment, significance was assessed by analysis of variance followed by Student’s t test, Dunnett’s test, and Tukey’s test. The accepted level of significance was \(P < 0.05\). For in vivo PET study, comparisons were performed by one-factor ANOVA. In the case of significant differences, post-hoc Tukey’s tests were performed. Statistical calculations were performed with GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA). Differences were considered significant at \(P < 0.05\).

**Results**

**Uptake of DPV and Pravastatin by Freshly Prepared Rat Hepatocytes.** The uptake of E217G (0.2 μM), a representative substrate of hepatic organic anion transporters, by the hepatocytes prepared for 5 minutes were 220 ± 4 and 21.9 ± 0.8 μl/mg protein in the presence and absence of rifampicin (Fig. 2A, inset), respectively, showing that the hepatocytes maintained sufficient uptake activity of anionic drugs as compared with that in previous reports (Ishizuka et al., 1998). Both DPV and pravastatin accumulated in freshly isolated rat hepatocytes with increasing incubation time (Fig. 2A). Edie-Hofstee plot shows one saturable component, and one saturable and one nonsaturable component for DPV and pravastatin uptake, respectively (Fig. 2B). \(K_m\) (μM) and \(V_{\text{max}}\) (pmol/ min per milligram protein) of DPV were 19.9 ± 0.8 and 62, respectively, whereas \(K_m\) (μM), \(V_{\text{max}}\) (pmol/min per milligram protein), and the clearance for the nonsaturable component (μl/min per milligram protein) of pravastatin were 8.06 ± 3.23, 283 ± 107, and 4.88 ± 1.44, respectively. The \(K_m\) value of pravastatin determined in this study was somewhat smaller than the previously reported value (29 μM) (Yamazaki et al., 1993). Rifampicin significantly inhibited the uptake of both compounds with similar inhibition potency (Fig. 2C).

**Uptake of DPV and Pravastatin by rMrp2-Expressing Membrane Vesicles.** As a positive control, the uptake of E217G was determined in rMrp2-expressing membrane vesicles. The uptake of DPV and pravastatin by rMrp2-expressing vesicles was slightly higher in the presence of ATP than that in the presence of AMP, showing statistical significance (Fig. 3).
The uptake of E217βG was also determined in the presence of compounds (100 μM each) to evaluate the magnitude of saturation of rMrp2 at the concentration employed. The uptake in the presence of ATP decreased to 56 and 71% in the presence of DPV and pravastatin, respectively (data not shown).

**Biodistribution of Radioactivity in the Abdominal Region after Intravenous Administration of [11C]DPV**

We previously reported a preliminary PET imaging study with [11C]DPV in rats (Ijuin et al., 2012). The time profiles of the distribution of radioactivity in the abdominal region are shown in Supplemental Movie 1. Consistent with this previous report, the radioactivity was localized primarily in the liver and kidneys, and distribution in other organs was not significantly observed in the control and rifampicin-treated (intravenous infusion at 1.5 μmol/min per kilogram) rats, as well as EHBR immediately after administration. Subsequently, the radioactivity gradually disappeared from the liver and kidneys, with most of it being transferred into the intestinal lumen, mainly by biliary excretion, in the control rat (68 ± 9% of the injected dose).

A separate conventional biodistribution study was conducted in the control rats (n = 3–4). The radioactivity (mean ± S.D.) distributed in the liver (% of the injected dose) were 19 ± 13, 21 ± 2, 14 ± 3, 12 ± 4, and 9 ± 8 at 5, 10, 20, 30, and 60 minutes after administration, respectively. The cumulative radioactivity excreted into bile (% of the injected dose) were 11 ± 6, 34 ± 13, 59 ± 14, and 78 ± 9 at 5, 10, 20, and 30 minutes, respectively, after administration. Meanwhile, the corresponding radioactivity obtained from PET imaging analysis in the control rats (n = 3) were 21 ± 2, 14 ± 2, 8 ± 2, 6 ± 2, and 4 ± 1 in the liver at 5, 10, 20, 30, and 60 minutes, respectively. The radioactivity that accumulated in the liver according to the PET data were slightly lower than the actual values, possibly because of the lag of time-points, as well as a partial volume effect on the PET data.


Figure 4A shows representative TLC autoradiograms of extracts from the blood 20 minutes after the administration of [11C]DPV to the control and rifampicin-treated rats, as well as EHBR. TLC analysis revealed that at least one identifiable metabolite (M1) and other metabolites were observed with [11C]DPV in the blood in all groups. The abundance of parent [11C]DPV in the blood (% of the total activity applied) in the control rats (n = 3) decreased with time: 97 ± 1 at 2 minutes, 68 ± 15 at 5 minutes, 55 ± 11 at...
Time-Plasma $^{11}$C]DPV Concentration Profiles and Tissue Distribution after Intravenous Administration of $^{11}$C]DPV in Rats. The total radioactivity in the blood samples is shown in Fig. 5A. The radioactivity was rapidly eliminated from the blood, showing biphasic profiles in the control rats. The radioactivity also decreased rapidly in both rifampicin-treated rats and EHBRs by 5 minutes, but thereafter the elimination was significantly delayed. In the liver, a maximum of $28 \pm 5\%$ of the injected dose was distributed within 2 minutes of administration, and then the radioactivity was eliminated until the end of the PET scan in the control rats. The AUCs of radioactivity in the liver were 1.8- and 3.0-fold larger in the rifampicin-treated rats and EHBRs than in the control rats (Fig. 5B). The profiles of radioactivity in the intestine were regarded as those excreted into bile, although the possibility that part of the radioactivity was absorbed at the intestine or secreted into the lumen from the blood circulation during PET scan cannot be ruled out. The radioactivity increased in the control rat to 60% within 20 minutes of administration, and finally reached $68 \pm 9\%$ of the injected dose by 60 minutes. The biliary excretion of radioactivity was dramatically reduced in both rifampicin-treated rats and EHBRs as compared with that in the control rats. Their maximum amounts of the injected dose were $24 \pm 6\%$ and $15 \pm 2\%$ within 30 minutes of administration, respectively (Fig. 5C). The radioactivity profiles in the kidneys are shown in Fig. 5D. The levels of radioactivity in the kidneys of the control and rifampicin-treated rats, as well as EHBRs, were similar: $11 \pm 2\%$, $8 \pm 1\%$, and $10 \pm 1\%$ of the injected dose within 1 minute, respectively. However, the subsequent elimination was delayed in the rifampicin-treated rats and EHBRs compared with that in the control rats (Fig. 5D). The plasma concentration of rifampicin was determined by only a single replicate rat and was $80$ and $93 \mu M$, before and after PET scan, respectively. These values were somewhat lower but comparable to our reported value (101–118 \mu M, Takashima et al., 2011a). The unbound fraction of rifampicin in control rat plasma was determined in vitro to be $0.137 \pm 0.017$ (mean $\pm$ S.E.; $n = 3$).

Liver Uptake and Canalicular Efflux Clearance of $^{11}$C]DPV. Representative maximum intensity projection PET images of the radioactivity in the abdominal region over the duration of the integration plot analysis for $Cl_{uptake,liver}$ and $Cl_{uptake,kidney}$, as well as $Cl_{int,bile}$, are shown in Fig. 6, A and B, respectively. The plots for $Cl_{uptake,liver}$, $Cl_{int,kidney}$, and $Cl_{int,bile}$ are shown in Fig. 6, C–E, respectively. Pharmacokinetic parameters are summarized in Table 1. Linearity of the plot was achieved only for a short period between 0.3 and 0.9 minutes for the liver and kidney uptake (Fig. 6, C and D). $Cl_{uptake,liver}$ of $^{11}$C]DPV was similar to the hepatic blood flow rate (55 ml/min per kilogram), and $Cl_{uptake,kidney}$ was two-thirds of the renal blood flow rate (37 ml/min per kilogram) (Davies and Morris, 1993). Both $Cl_{uptake,liver}$ and $Cl_{uptake,kidney}$ decreased to approximately 69 and 87% of the control values in rifampicin-treated rats and EHBRs, respectively, although the difference between the control rats and EHBRs did not reach statistical significance.

The integration plot for $Cl_{int,bile}$ of radioactivity is shown in Fig. 6E. The $Cl_{int,bile}$ of radioactivity was $12.2 \pm 1.5$ ml/min per kilogram in the control rats, but was significantly lower at $5.0 \pm 1.6$ and $1.4 \pm 0.4$ ml/min per kilogram in rifampicin-treated rats and EHBRs, respectively (Table 1). Intrinsic
renal clearance with regard to the kidney and urinary bladder concentration was not measured in this study due to the limitation of a narrow field of view to visualize the whole urinary bladder on microPET.

**Discussion**

In the present study, we investigated pharmacokinetic properties of a newly developed PET tracer, [11C]DPV, for hepatobiliary transport in rats for future clinical investigations. For this purpose, in vivo hepatobiliary transport of [11C]DPV was determined in rats treated with rifampicin and Mrp2-deficient EHBR. In addition, metabolite assessment in rats in vivo and metabolic stability of [11C]DPV in humans using cryopreserved human hepatocytes in vitro was also demonstrated.

The uptake activity of DPV by freshly prepared rat hepatocytes in vitro was similar to that of pravastatin (Fig. 2A). The difference in the kinetic parameters was at most 2-fold. An Oatp inhibitor, rifampicin completely inhibited the uptake of both DPV and pravastatin, with similar inhibition potency (Fig. 2C). Three Oatp1 isoforms, Oatp1a1, Oatp1a4, and Oatp1b2, which are expressed in the sinusoidal membrane of hepatocytes, were all shown to be inhibited by rifampicin, with $K_i$ values of 18, 1.4–2.9, and 0.79 μM, respectively (Fattinger et al., 2000; Shitara et al., 2002; Lau et al., 2006). Complete inhibition of the uptake by rifampicin at the concentrations examined suggests that Oatps mediate the hepatic uptake of DPV and pravastatin. Because the uptake was significantly inhibited by rifampicin, even at 1 μM, similar to the $K_i$ values for Oatp1a4 and Oatp1b2, these transporters are candidates of the transporters responsible. In fact, Oatp1b2-null and Oatp1a1b-null mice manifest a significant reduction in the liver-to-plasma ratio of pravastatin (Zaher et al., 2008; Iusuf et al., 2012).

rMrp2-expressing membrane vesicles showed ATP-dependent uptake of DPV and pravastatin (Fig. 3), indicating that DPV is an rMrp2 substrate. Low transport activity of pravastatin by rMrp2 is consistent with that observed in the rat canaliculur membrane vesicles (CMVs). The ATP-dependent uptake of pravastatin by CMVs was one-fifth of that of E217βG, a representative Mrp2 substrate (Yamazaki et al., 1997; Morikawa et al., 2000). Saturation is another factor for low ATP-dependent uptake in rMrp2-expressing vesicles. Considering that the $K_m$ value of pravastatin for ATP-dependent uptake by CMV was 220 μM (Yamazaki et al., 1997), rMrp2 likely showed a moderate saturation at the concentration employed. In fact, the uptake of E217βG decreased to 56 and 70% of the control in the presence of DPV and pravastatin, respectively.

The liver and kidney were major organs for the distribution of [11C]DPV, and most of the radioactivity was recovered in the intestine via biliary excretion. The liver exhibited extensive tissue uptake, which was blood-flow–limited. Yet in the kidney, tissue uptake was likely similar to the renal blood-flow rate. Tissue uptake clearance could not explain the elimination pathway of [11C]DPV from the systemic circulation; biliary excretion is the predominant pathway. Oatps in the kidney, such as Oatp1a1 and Oatp1a6, are believed to mediate the reabsorption of amphipathic anionic compounds in males (Gotoh et al., 2002; Chung et al., 2005). Negligible urinary excretion of [11C]DPV, despite extensive renal uptake from the blood circulation, may be attributable to its reabsorption from the urine.

Rifampicin and deficiency of Mrp2 markedly reduced the biliary excretion of radioactivity, and consequently increased the systemic and liver exposure of [11C]DPV (Fig. 5, A–C), and the underlying mechanism of this increase may be elucidated by the determination of the tissue uptake and canalicular eflux clearances. Deficiency of Mrp2 did not affect CLuptake,liver.
The administration of rifampicin caused only a slight decrease in this parameter, although rifampicin could inhibit Oatps based on the in vivo and in vitro data; taking the unbound fraction of rifampicin in rat plasma, the unbound concentration in this study was in the range of 11–13 mM, high enough to produce a significant inhibition of Oatps based on its $K_i$ values for Oatps and IC$_{50}$ observed in isolated rat hepatocytes. The discrepancy between the in vitro and in vivo inhibition potency of rifampicin is attributable to the blood-flow-limited hepatic uptake of [$^{11}$C]DPV. In contrast, CL$_{int,bile}$ of [$^{11}$C]DPV was significantly decreased by both rifampicin treatment and Mrp2 deficiency, which accounted for the delay in the systemic elimination of [$^{11}$C]DPV. A marked reduction in CL$_{int,bile}$ in EHBR indicates a predominant role of Mrp2 in the canalicular efflux of [$^{11}$C]DPV in rats. The inhibition of Mrp2 by rifampicin at the dose examined was also suggested by the use of another PET ligand, 15R-[$^{11}$C]TIC, the biliary excretion of which was also mainly

**TABLE 1**
Pharmacokinetic parameters of [$^{11}$C]DPV after intravenous administration in the control and rifampicin-treated rats and EHBRs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Rifampicin-Treated</th>
<th>EHBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-60min,blood}$ (% dose·min/ml)</td>
<td>8.6 ± 4.8</td>
<td>14.9 ± 1.5</td>
<td>11.0 ± 1.6</td>
</tr>
<tr>
<td>CL$_{uptake,liver}$ (ml/min per kilogram)</td>
<td>73.6 ± 4.8</td>
<td>50.8 ± 6.9*</td>
<td>64.3 ± 8.0</td>
</tr>
<tr>
<td>$V_E$$_{liver}$ (ml per kilogram)</td>
<td>4.1 ± 4.6</td>
<td>4.5 ± 2.0</td>
<td>2.2 ± 2.4</td>
</tr>
<tr>
<td>CL$_{uptake,kidney}$ (ml/min per kilogram)</td>
<td>24.6 ± 0.6</td>
<td>17.0 ± 2.3*</td>
<td>21.2 ± 2.7</td>
</tr>
<tr>
<td>CL$_{uptake,kidney}$ (ml/min per kilogram)</td>
<td>6.4 ± 0.8</td>
<td>5.2 ± 0.7</td>
<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>$V_E$$_{kidney}$ (ml per kilogram)</td>
<td>12.2 ± 1.5</td>
<td>5.0 ± 1.6**</td>
<td>1.4 ± 0.4***</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001 versus control rat.
mediated by Mrp2 (Takashima et al., 2010). The fact that \( C_{\text{int,bile}} \) of \([^{11}\text{C}]\text{DPV}\) in rifampicin-treated rats remained higher than that in EHRBR (Fig. 6E) indicates moderate inhibition of Mrp2 at this dose. Our clinical investigation using 15R-[\( ^{11}\text{C} \)]TIC-Me demonstrated that the effect of rifampicin on \( C_{\text{int,bile}} \) as well as \( C_{\text{uptake,liver}} \) could be reproduced in humans (Takashima et al., 2012). Thus, we speculate that the DDIs with rifampicin may involve both OATP and MRp2 inhibition in the hepatobiliary transport of anionic drugs. Bile salt export pump ABCB11 may be partly involved in the biliary excretion of \([^{11}\text{C}]\text{DPV}\) in humans. Pravastatin was found to be a substrate of bile salt export pump (Hirano et al., 2005), whereas the transport activities of pravastatin by other canalicular drug transporters such as MDR1 and BCRP was fairly low compared with MRp2 (Matsushima et al., 2005). For the evaluation of the contribution of MRp2 to the canalicular efflux of \([^{11}\text{C}]\text{DPV}\) in the human liver, it is necessary to interpret the clinical PET data of DDI.

Notably, the radioactivity in the liver included contributions from radioactivity in blood and bile as well as that associated with hepatocytes where the tissue uptake and canalicular efflux occurred. According to the principle of the integration plot analysis, it is not necessary to correct the contamination of the radioactivity associated with blood in integration plot analysis, it is not necessary to correct the contribution from radioactivity in blood and bile, since measurement of this parameter theoretically requires the radioactivity in the hepatocytes where the canalicular efflux occurred.

Extensive metabolism in the liver obviously precludes the quantification of \( C_{\text{int,bile}} \) of PET probes. The extent of DPV metabolism in the human liver is a critical issue to consider when applying it to measure canalicular efflux. Unlike rats, in vitro radiometabolite analysis using cryopreserved human hepatocyte suspension showed negligible metabolism of \([^{11}\text{C}]\text{DPV}\) for 90-minutes incubation (Fig. 4A). This holds true for pravastatin; it undergoes significant biotransformation in rat liver, whereas it is hardly metabolized in humans (Beaird, 2000; Watanabe et al., 2009). Therefore, it is highly possible that \([^{11}\text{C}]\text{DPV}\) is a nonmetabolizable PET probe for hepatobiliary transport in humans.

Blood-flow–limited tissue uptake also precluded the quantification of OATP activities. There is a great difference in hepatic elimination between rats and humans. For example, hepatic uptake clearance of 15R-[\( ^{11}\text{C} \)]TIC in rat was close to the hepatic blood-flow rate (Takashima et al., 2010), but not in humans (Takashima et al., 2012). This holds true for pravastatin, in which the hepatic uptake and overall elimination is hepatic blood-flow–limited in rats (Yamazaki et al., 1996), whereas the nonrenal clearance of pravastatin (7.2 ml/min per kilogram; Singhi et al., 1990) is one-third of the hepatic blood-flow rate (20.7 ml/min per kilogram) in humans. Therefore, it is highly possible that the hepatic elimination of \([^{11}\text{C}]\text{DPV}\) is not blood-flow–limited in humans.

Pharmacokinetic analysis involving tissue concentration–time profile using \([^{11}\text{C}]\text{DPV}\) should elucidate the mechanisms of DDI in hepatobiliary transport, particularly in the canalicular efflux process by MRp2. For instance, rifampicin and probenecid are considered to inhibit MRp2 at clinical doses (Horikawa et al., 2002; Takashima et al., 2012). Rifampicin causes an increase in the systemic exposure of pravastatin in humans (Kyrklund et al., 2004). Clinical DDI study using \([^{11}\text{C}]\text{DPV}\) should elucidate the magnitude of interaction with rifampicin or probenecid in the canalicular efflux process. Moreover, \([^{11}\text{C}]\text{DPV}\) should be useful in pharmacogenomic studies. Genotypes of OATP1B1 are associated with interindividual differences in the systemic exposure of statins (Nishizato et al., 2003; Chung et al., 2005; Lee et al., 2005; Pasanen et al., 2006, 2007). Another report has described the association of single nucleotide polymorphisms of MRp2 with the plasma levels of its substrates, phase II metabolites of isoflavonoids (Kato et al., 2012), or adverse reactions to drugs (Kim et al., 2010). \([^{11}\text{C}]\text{DPV}\) will provide quantitative information on the effect of single nucleotide polymorphisms on OATP1B1 and MRp2 activities. Furthermore, integration of quantitative data for the tissue distribution and plasma concentration–time profile of PET probe using a physiologically based pharmacokinetic model is a promising approach to estimate all the clearances of PET probe, including the sinusoidal efflux clearance which cannot be directly measured, when the biliary excretion and hepatic metabolism data are acquired. This should help further understanding of mechanisms and factors affecting the clearance of PET probes.

In conclusion, we demonstrated the utility of \([^{11}\text{C}]\text{DPV}\) for noninvasive PET imaging of hepatobiliary transport involving Oatps and Mrp2 in rat. Because of its negligible metabolism in human hepatocytes, \([^{11}\text{C}]\text{DPV}\) will be a good probe for studying the function of Oatps and MRp2 in future clinical settings.

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**Authorship Contributions**

**Participated in research design:** Shingaki, Takashima, Maeda, Kusuhara, Watanabe.

**Conducted experiments:** Shingaki, Okauchi, Takashima, Zhang, Onoue, Katayama.

**Contributed new reagents or analytic tools:** Ijuin, Katayama, Okachi, Hayashinaka, Wada.

**Performed data analysis:** Shingaki, Takashima, Zhang, Onoue, Katayama.

**Wrote or contributed to the writing of the manuscript:** Shingaki, Takashima, Cui, Wada, Suzuki, Maeda, Kusuhara, Sugiyama.

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Address correspondence to: Dr. Yasuyoshi Watanabe, RIKEN Center for Life Science Technologies, 6-7-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan. E-mail: yywatan@riken.jp