Blood-Brain Barrier Pharmacoproteomics-Based Reconstruction of the In Vivo Brain Distribution of P-Glycoprotein Substrates in Cynomolgus Monkeys

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ABSTRACT

The aim of this study was to investigate whether in vivo drug distribution in brain in monkeys can be reconstructed by integrating four factors: protein expression levels of P-glycoprotein (P-gp)/multidrug resistance protein 1 at the blood-brain barrier (BBB), in vitro transport activity per P-gp molecule, and unbound drug fractions in plasma and brain. For five P-gp substrates (indinavir, quinidine, loperamide, paclitaxel, and verapamil) and one non-substrate (diazepam), in vitro P-gp transport activities were determined by measuring transcellular transport across monolayers of cynomolgus monkey P-gp-transfected LLC-PK1 and parental cells. In vivo P-gp functions at the BBB were reconstructed from in vitro P-gp transport activities and P-gp expression levels in transfected cells and cynomolgus brain microvessels. Brain-to-plasma concentration ratios (Kp,brain) were reconstructed by integrating the reconstructed in vivo P-gp functions with drug unbound fractions in plasma and brain. For all compounds, the reconstructed Kp,brain values were within a 3-fold range of observed values, as determined by constant intravenous infusion in adult cynomolgus monkeys. Among four factors, plasma unbound fraction was the most sensitive factor to species differences in Kp,brain between monkeys and mice. Unbound brain-to-plasma concentration ratios (Kp,uu,brain) were reconstructed as the reciprocal of the reconstructed in vivo Kp,brain functions, and the reconstructed Kp,uu,brain values were within a 3-fold range of in vivo values, which were estimated from observed Kp,brain and unbound fractions. This study experimentally demonstrates that brain distributions of P-gp substrates and nonsubstrate can be reconstructed on the basis of pharmacoproteomic concept in monkeys, which serve as a robust model of drug distribution in human brain.

Introduction

The number of compounds approved for use as new drugs is very small compared with the number of drug candidates that progress from preclinical to clinical trials. The proportion was just 8% for central nervous system (CNS)–acting drugs during the period 1991–2000 (Kola and Landis, 2004), and also no greater during the period 2000–2008 (Yagi and Ohkubo, 2010). One of the major reasons for the high rate of discontinuation has been the unfavorable distribution of drugs into human brain. More than 98% of small molecules do not cross the blood-brain barrier (BBB) and, thus, do not provide pharmacologically active concentrations in brain (Partridge, 2002). It has been also reported that there are 7638 molecules in the Comprehensive Medicinal Chemistry database but only 387 (5.1%) of these molecules treat CNS diseases (Ghose et al., 1999). Therefore, it is necessary to make a quantitative and accurate prediction of drug distribution in human brain during the preclinical stages. Brain drug distribution depends on permeability rate across the BBB regulated by a variety of transporters expressed in brain capillary endothelial cells. ATP-binding cassette (ABC) transporters P-glycoprotein just 8% for central nervous system (CNS)–acting drugs during the period 1991–2000 (Kola and Landis, 2004), and also no greater during the period 2000–2008 (Yagi and Ohkubo, 2010). One of the major reasons for the high rate of discontinuation has been the unfavorable distribution of drugs into human brain. More than 98% of small molecules do not cross the blood-brain barrier (BBB) and, thus, do not provide pharmacologically active concentrations in brain (Partridge, 2002). It has been also reported that there are 7638 molecules in the Comprehensive Medicinal Chemistry database but only 387 (5.1%) of these molecules treat CNS diseases (Ghose et al., 1999). Therefore, it is necessary to make a quantitative and accurate prediction of drug distribution in human brain during the preclinical stages. Brain drug distribution depends on permeability rate across the BBB regulated by a variety of transporters expressed in brain capillary endothelial cells. ATP-binding cassette (ABC) transporters P-glycoprotein...
(P-gp)/multidrug resistance protein 1 (MDR1/mdr1a/ABCB1 and breast cancer resistance protein (BCRP)/ABCG2 are major gatekeepers for many drugs (Kusuhara and Sugiyama, 2009; Uchida et al., 2011b). Therefore, it is important to quantitatively clarify the molecular functions of these transporters at the human BBB to predict drug distribution in the human brain.

Protein expression levels have been reported to correlate with activities of functional proteins (Dyer et al., 1997; Hoffmeyer et al., 2000; Fukumoto et al., 2002; Shirasaka et al., 2008; Langenfeld et al., 2009; Tachibana et al., 2010). Hence, we anticipated that in vivo functional activities of target transporters could be reconstructed on the basis of their in vitro activities by integrating these activities with the in vivo/in vitro differences in protein expression levels. We developed an absolute protein quantification method for transporters that uses liquid chromatography–tandem mass spectrometry (LC-MS/MS), termed “quantitative targeted absolute proteomics (QTAP)” (Kamie et al., 2008). Using QTAP, we demonstrated in mouse model that in vivo P-gp/mdr1a function at the BBB was reconstructed by integrating the protein expression levels of P-gp/mdr1a in the in vivo brain capillaries with the transport function per P-gp/mdr1a molecule, which was determined using an in vitro transport experiment and QTAP (Uchida et al., 2011a). This demonstration opened a new field of pharmacoproteomics (PPx) that is an integrated scientific field of proteomics and pharmacokinetics/pharmacodynamics/toxicokinetics/toxicodynamics to quantitatively understand drug absorption, distribution, metabolism, and excretion; pharmacologic effect; and toxicity (Uchida et al., 2014).

However, brain distributions of P-gp substrates, such as [18F]altanserin and [11C]GR205171 (S)-(2-methyl-5-(5-trifluoromethyltetrazol-1-yl)-phenylmethylamino)-2-(S)-phenylpiperidine, significantly differ between humans and rodents (Syvanen et al., 2009). Therefore, it is debatable whether the demonstration of in vitro–to–in vivo reconstruction (IVIVR) in a mouse model alone is sufficiently valid to apply the theory to reconstruction of in vivo P-gp function at the human BBB, although this reconstruction is theoretically applicable regardless of animal species. In contrast, the quantitative protein expression profile of BBB transporters in cynomolgus monkeys is quite similar to that in humans (e.g., only 1.29-fold difference from humans in P-gp protein levels) (Ito et al., 2011; Uchida et al., 2011b).

It is also important to overcome species difference in brain drug distribution. Brain-to-plasma concentration ratios ($K_{\text{p,brain}}$) of P-gp substrate verapamil and PF-00905556 in monkey are significantly, 10.8- and 12.2-fold, greater than those in mouse although this reconstruction is theoretically applicable regardless of animal species. In contrast, the quantitative protein expression profile of BBB transporters in cynomolgus monkeys is quite similar to that in humans (e.g., only 1.29-fold difference from humans in P-gp protein levels) (Ito et al., 2011; Uchida et al., 2011b).

The purpose of this study was to experimentally demonstrate the reconstruction/prediction theory in cynomolgus monkeys to ensure that the theory is applicable for clarifying in vivo human BBB P-gp function and predicting brain drug distributions in humans. We reconstructed the brain distributions ($K_{\text{p,brain}}$ and $K_{\text{p,uu,brain}}$) of six model compounds including five P-gp substrates and one nonsubstrate in cynomolgus monkeys on the basis of the theory that was previously established in a mouse model, and compared them with the observed brain distributions determined in an in vivo study to validate whether in vivo P-gp function at the BBB in monkeys could be reliably reconstructed. Furthermore, we analyzed the influence of species differences in four factors (BBB P-gp protein expression levels, intrinsic transport activity per P-gp molecule, and unbound fractions in plasma and brain) on species differences in brain distributions of P-gp substrates between mice and monkeys.

Materials and Methods

Chemicals. Buspirone hydrochloride, loperamide hydrochloride, and quinine were purchased from Sigma-Aldrich (St. Louis, MO). Diazepam, paclitaxel, and verapamil hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan). Indinavir sulfate was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). P-gp peptides with >95% peptide purity were synthesized by Thermo Fisher Scientific (Sedanstrasse, Germany). All of the other chemicals were of reagent grade and were available commercially.

Animals. Six male adult cynomolgus monkeys were used for the intravenous constant infusion study and the absolute quantification of P-gp protein expression in isolated brain microvessels. The animals were treated as follows: 1) aged 5 years and 2 months with 3.6 kg b.wt. was treated with indinavir; 2) aged 4 years 1 month with 2.6 kg b.wt. was treated with quinidine; 3) aged 6 years 4 months with 5.4 kg b.wt. was treated with verapamil; 4) aged 5 years 4 months with 3.4 kg b.wt. was treated with paclitaxel; 5) aged 4 years 3 months with 3.15 kg b.wt. was treated with diazepam; and 6) aged 4 years 1 month with 3.2 kg b.wt. was treated with verapamil. The six cynomolgus monkeys received intravenous constant infusions of the test compounds after fasting overnight with free access to water at HAMRI, Co., Ltd. (Ibaraki, Japan), after which the right cerebrums were used to determine the compound concentrations in the cerebrums and the left cerebrums were used for the P-gp quantifications in the brain microvessels.

Another male adult cynomolgus monkey, aged 4 years 1 month with 3.3 kg b.wt., was used as a blank control for the intravenous constant infusion study. The control cynomolgus monkey underwent the same procedure as the six cynomolgus monkeys outlined above without compound administration at HAMRI, Co., Ltd. The plasma and cerebrum of the control cynomolgus monkey were used as blank samples for the concentration determinations of the six test compounds in the plasma and cerebrum, respectively. The cerebrum of the control animal was also used for the measurements of the unbound fractions in the cerebrum.

Animal care and experimental procedures for the cynomolgus monkeys were approved by the Animal Care and Use Committee of Banyu Tsukuba Research Institute.

Determination of the Brain Distributions of the Six Test Compounds in Cynomolgus Monkeys. After fasting overnight with free access to water, the male adult cynomolgus monkeys were fixed to monkey chairs and received constant infusions of the test compounds (indinavir, quinidine, loperamide, paclitaxel, diazepam, or verapamil) via the cephalic or saphenous veins for 3 hours without anesthesia. With the exception of leoperamide and paclitaxel, intravenous bolus injections of the test compounds were performed immediately prior to the constant infusion to quickly reach the steady-state plasma...
transplantation; indinavir, quinidine, diazepam, and verapamil were intravenously infused for 3 hours at dose rates of 0.61, 0.21, 0.070, and 0.40 mg/kg per kilogram, respectively, after the intravenous bolus injection of 0.50, 0.30, 0.095, and 1.2 mg/kg doses. Loperamide and paclitaxel were intravenously infused for 3 hours at dose rates of 0.20 and 0.94 mg/kg per kilogram, respectively, without intravenous bolus injections. One cynomolgus monkey was used for each compound. Prior to administration and at 2, 2.5, and 3 hours after administration, blood samples were collected using syringes containing EDTA–dipotassium salt (2K) without anesthesia from the cephalic or saphenous vein on the side opposite that used for administration. The blood samples were immediately centrifuged at 4°C and 3500g for 10 minutes to obtain plasma. The plasma samples were stored at −80°C prior to LC-MSMS analyses. Immediately after the blood sampling at 3 hours, the cynomolgus monkeys were sacrificed by exsanguination under anesthesia with isoﬂurane inhalation or excessive anesthesia with pentobarbital without exsanguination, and the brains were immediately excised, divided into the right and left cerebrums (for the P-gp quantifications in the brain microvessels), frozen in liquid nitrogen, and stored at −80°C prior to the LC-MS/MS analyses. For the LC-MS/MS analyses, the right cerebrums were weighed and homogenized with a 2-fold volume of phosphate-buffered saline (PBS) to obtain a 33.3% brain homogenate. Ten microliters of plasma or brain homogenate was mixed with 100 μl of ethanol and 100 μl of 75% ethanol containing buspirone as an internal standard (210 μl in total). The samples were centrifuged and filtrated at 4°C and 960g for 10 minutes and subjected to LC-MS/MS analyses. On the basis of pharmacokinetic concepts, the brain-to-plasma concentration ratio of the test compound (K \text{brain-to-plasma}) was estimated by dividing the cerebral concentrations by the plasma concentrations. **Transcellular Transport Study across Cynomolgus Monkey P-gp-Transfected LLC-PK1 and Parental LLC-PK1 Cell Monolayers.** Cynomolgus monkey P-gp–transfected LLC-PK1 and parental LLC-PK1 cells were prepared at Merck Research Laboratories (West Point, PA) (Sankaranarayanan et al., 2009). The transcellular transport study was carried out as described previously (Uchida et al., 2011a) with minor modifications. Cynomolgus monkey P-gp–transfected LLC-PK1 and parental LLC-PK1 cells were seeded at a density of 0.15 × 10⁶ cells/insert (0.484 ml) in 12-well plates (BD Biosciences) that had been coated with BD Matrigel Basement Membrane Matrix. The cells were cultured under the same conditions as those used in the transcellular transport study, and the cells were used for the experiments on the fourth day after seeding. Hanks’ balanced salt solution containing HEPES (136.7 mM NaCl, 5.36 mM KCl, 0.952 mM CaCl₂, 0.812 mM MgSO₄, 0.441 mM KH₂PO₄, 0.385 mM Na₂HPO₄, 25 mM t-glucose, 10 mM HEPES, pH 7.2–7.4) was used as a transport buffer and used throughout the transport experiments. The cell monolayers were preincubated in the transport buffer without test compounds at 37°C for approximately 1 hour, and then the transport experiments were initiated by replacing the buffer in each compartment with 0.5 ml of fresh transport buffer with (donor compartment) and without (acceptor compartment) the test compounds (37°C). Six compounds were tested at 0.5 μM (indinavir, quinidine, loperamide, diazepam, and verapamil) or 1 μM (paclitaxel) concentrations. Both the preincubation and transport experiments were performed in a 5% CO₂ incubator at 37°C. For basal-to-apical transport, the short interval (10 minutes) between sampling times (10, 20, and 30 minutes after initiation) was selected to maintain the concentration in the donor compartment sufficiently higher than that in the acceptor compartment to prevent the underestimation of donor-to-acceptor transport. At each sampling time, 100-μl aliquots were taken from the apical side, the culture inserts (apical side) were transferred to new (vacant) wells of a 24-well plate that had been prewarmed at 37°C, and 0.5 ml of fresh transport buffer with and without the test compounds (37°C) was added to the basal and apical sides, respectively, to maintain sink conditions. For apical-to-basal transport, 100-μl aliquots were taken from the basal side at each sampling time (30, 60, and 90 minutes), the culture inserts (apical side) were transferred to new (vacant) wells of a 24-well plate that had been prewarmed at 37°C, and 0.5 ml of fresh transport buffer (37°C) was added to the basal side to maintain sink conditions. One hundred microliters of acetonitrile containing buspirone (internal standard) was added to 100 μl of the collected samples, and the mixtures were then subjected to LC-MSMS analyses to quantify the amounts of the test compounds that had been transported to the acceptor side. The transported amounts (pmol/well) were plotted against the transport time (min), and then the transport rate (pmol/min per well) was obtained by the linear regression of three sampling time points. The flux ratio was obtained by dividing the transport rate in the basal-to-apical direction by that in the apical-to-basal direction. The flux ratio in the cynomolgus monkey P-gp–transfected LLC-PK1 cells was divided by that in the parental LLC-PK1 cells to obtain the in vitro P-gp efflux ratio. This in vitro P-gp efflux ratio was used as a measure of the in vitro P-gp transport activity. The paracellular flux was monitored in terms of the appearance of dextran Texas Red in the opposite compartment and was less than 2.6% of the total amount of dextran Texas Red. **Determination of Protein Expression Levels of Cynomolgus Monkey P-gp in the Cynomolgus Monkey P-gp-Transfected LLC-PK1 Cell Monolayer and Isolated Cynomolgus Monkey Brain Microvessels.** Cynomolgus monkey P-gp–transfected LLC-PK1 cells were seeded at a density of 2.09 × 10⁶ cells/insert (0.484 × 10⁶ cells/cm²), which is the same density used in the transcellular transport study, on porous (1.0-μm) polyethylene terephthalate membrane filters (cell culture inserts for six-well plates; BD Biosciences) that had been coated with BD Matrigel Basement Membrane Matrix. The cells were cultured under the same conditions as those used in the transcellular transport study, and the cells were used for the experiments on the fourth day after seeding. The apical and basal sides were washed with ice-cold PBS twice, and the cells from six sides were used to obtain a whole-cell lysate. Cynomolgus monkey brain microvessels were isolated from the left cerebrums that had been excised from the cynomolgus monkeys used for the in vivo constant infusion study. The microvessels were isolated by using a combination of dextran density gradient separation and size filtration (nylon mesh method). The isolation procedure was the same as that described in Ito et al. (2011). The protein expression levels of cynomolgus monkey P-gp in the whole-cell lysates of cynomolgus monkey P-gp–transfected LLC-PK1 cells and the whole-tissue lysates of the isolated cynomolgus monkey brain microvessels were determined by using the same procedure as that described in Ito et al. (2011). **Determination of the Unbound Fractions in Cynomolgus Monkey Plasma Using the Equilibrium Dialysis Method.** Cynomolgus monkey plasma (KAC Co., Ltd., Kyoto, Japan) samples were adjusted to pH 7.4 with saturated CO₂ prior to sample preparation. Five microliters of 50% acetonitrile-containing test compound (50 μM) was added to 495 μl of the plasma to obtain a 0.5 μM final concentration in the plasma. One hundred twenty microliters of PBS (pH 7.4) and 120 μl of plasma containing the compounds were placed in the dialysate and sample sides of the 96-well microequilibrium dialysis device (HTD 96b, HTDialysis, Gales Ferry, CT), respectively, with HTD 96a Dialysis Membrane Strips (molecular mass cut-off 12–14 kDa; HTDialysis). The wells were sealed and rotated in a 10% CO₂ incubator at 37°C and 80 rpm with a multishaker (TOKYO RIKAKIKA Co., Ltd., Tokyo, Japan). After a 6-hour incubation, 5 μl of plasma and 50 μl of dialysate were collected from the sample and dialysate sides, respectively, and transferred to 96-well format polypropylene plates. Fifty microliters of control PBS and 5 μl of control plasma were added to the collected plasma and dialysate
samples, respectively. One hundred fifty microliters of acetonitrile-containing buspirone (internal standard) was added (205 µl in total) and vortexed. The samples were centrifuged and filtered at 4°C and 900g for 10 minutes and subjected to LC-MS/MS analyses. The unbound fractions of the test compounds in the cynomolgus monkey plasma were calculated on the basis of the ratio of the concentrations that was determined from the plasma and dialysate samples.

**Determination of the Unbound Fractions in Cynomolgus Monkey Brain Using a Combination of the Homogenate Method and a pH Partition Model.** The unbound brain fractions were determined by equilibrium dialysis using the brain homogenates in combination with a pH partition model, as previously described (Friden et al., 2011) with minor modifications. The frozen cerebrum of the control cynomolgus monkey was used for this experiment. Cellulose membranes with a molecular mass cutoff of 14,000 Da were used. The samples were centrifuged and filtrated at 4°C and 300-g for 10 minutes. The supernatant (180 µl) and dialysate sample (205 µl) were collected from the apparatus. One hundred ninety microliters of acetonitrile-0.1% aqueous formic acid and centrifuged at 4°C and 17,360 g for 20 minutes, the samples were centrifuged at 4°C and 17,360 g. The supernatant (180 µl) and dialysate sample (205 µl) were added to 10 mM HEPES, pH 7.4, and conditioned in ECF buffer overnight. The cerebrum was diluted 4-fold with ECF buffer, homogenized using a sonic probe on ice, and then spiked with the test compounds. After preincubation at 37°C for 10 minutes, 400 µl of the brain homogenates containing the compounds (260 nM indinavir, 559 nM quinidine, 870 nM loperamide, 3110 nM paclitaxel, 333 nM diazepam, and 117 nM verapamil) were loaded into the chambers of a Sanplatec equilibrium dialysis apparatus (Osaka, Japan), mounted with the dialysis membranes, and dialyzed against 700 µl of ECF buffer that had been preincubated at 37°C. The equilibrium dialysis apparatus was incubated in a 37°C incubator for 6 hours with 300-rpm shaking. After 6 hours, the brain homogenates and dialysate samples were collected from the apparatus. One hundred ninety microliters of acetonitrile containing 1% formic acid and buspirone (internal standard) and 5 µl of 50% acetonitrile were added to 10 µl of the brain homogenate sample (205 µl in total). One hundred microliters of acetonitrile containing 1% formic acid and buspirone (internal standard) and 5 µl of 50% acetonitrile were added to 100 µl of the dialysate sample (205 µl in total). After having been shaken for 20 minutes, the samples were centrifuged at 4°C and 17,360 g for 5 minutes. The supernatant (180 µl) was then evaporated by centrifugation under vacuum. The residue was then reconstituted in 0.1% aqueous formic acid and centrifuged at 4°C and 17,360 g for 5 minutes. The supernatant (180 µl) was then evaporated by centrifugation under vacuum. The residue was then reconstituted in 0.1% aqueous formic acid and centrifuged at 4°C and 17,360 g for 6 hours. The dialysate and brain homogenate fractions were determined using the values of $f_{uu,brain}$ and $f_{brain}$ using eq. (1) and the reported $pK_a$ values of the test compounds (see Table 3 caption).

**LC-MS/MS Analyses of the Compounds.** The sample analyses were automated by coupling a triple quadrupole mass spectrometer (API4000 or API5000; AB SCIEX, Framingham, MA) to an Agilent 1200 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA). The samples were injected onto either an Agilent XDB-C18 column (2.1 × 150 mm, 5 µm) or a Shiseido Capcell Pak UG120 C18 column (2.0 × 150 mm, 5 µm). The compounds were separated and eluted from the columns under linear gradient or isocratic conditions with a flow rate of 0.2–0.3 ml/min. The eluted compounds were detected using electrospray ionization in selected/multiple reaction monitoring mode. Selected/multiple reaction monitoring transitions for indinavir, quinidine, loperamide, paclitaxel, diazepam, verapamil, and buspirone were 614.5/421.3, 325.3/172.3, 477.4/266.0, 854.5/569.3, 285.1/193.5, 455.1/165.3, and 386.3/122.3, respectively.

**IVIVR Theory of BBB P-gp Function and the Brain Distributions of P-gp Substrates.** According to the reconstruction theory reported in Uchida et al. (2011a), in vivo P-gp function at the cynomolgus monkey BBB and the brain distributions of P-gp substrates and nonsubstrate in cynomolgus monkeys were reconstructed using the in vitro experimental values.

In vivo P-gp function at the cynomolgus monkey BBB is defined as the $K_{p,brain}$ ratio, which is the ratio of $K_{p,brain}$ in P-gp knockout animals to that in wild-type animals. As previously described (Uchida et al., 2011a), the $K_{p,brain}$ ratio was reconstructed using eq. 2 and the in vitro P-gp efflux ratio and protein expression levels of P-gp in the cynomolgus monkey P-gp–transfected LLC-PK1 cell monolayer and isolated cynomolgus monkey brain microvessels.

$$K_{p,brain} = \frac{f_{plasma}}{f_{brain} \times K_{p,brain}}$$

Further, the $K_{p,uu,brain}$ is defined as follows:

$$K_{p,uu,brain} = \frac{C_{u,brain}}{C_{u,plasma}} = \frac{K_{p,brain} \times f_{brain}}{f_{plasma}}$$

The $K_{p,uu,brain}$ in cynomolgus monkeys was reconstructed as the reciprocal of the reconstructed $K_{p,brain}$ ratio in eq. 5, which was obtained using eqs. 3 and 4:

$$K_{p,uu,brain} = \frac{1}{K_{p,brain}}$$

We were unable to determine the observed $K_{p,brain}$ ratio in cynomolgus monkeys because we did not have a P-gp knockout cynomolgus monkey. However, the observed $K_{p,brain}$ and $K_{p,uu,brain}$ can be determined via in vivo experiments with wild-type cynomolgus monkeys and consist of the $K_{p,brain}$ ratio, as described in eqs. 3 and 5. Therefore, the reconstructions of the $K_{p,brain}$ and $K_{p,uu,brain}$ can be validated by comparing the reconstructed values with the observed values. The reconstruction of the $K_{p,brain}$ ratio can also be evaluated on the basis of the validation of the $K_{p,brain}$ and $K_{p,uu,brain}$ reconstructions, leading to the validation of IVIVR of P-gp function at the cynomolgus monkey BBB.

**Quantitative Evaluation of the Effects of Individual Parameters on Species Differences in K_{p,brain} and K_{p,uu,brain} between Cynomolgus Monkeys and Mice.** Using eqs. 2 and 3, the $K_{p,brain}$ is described by four parameters, as follows:

$$K_{p,brain} = \frac{f_{plasma}}{f_{brain} \times (1 + T_{int,pgp} \times P_{int,pgp,ivivo})}$$

where $P_{int,pgp,ivivo}$ represents the protein expression levels of P-gp in isolated brain microvessels and $T_{int,pgp}$ represents the intrinsic transport activity per P-gp molecule, which is calculated as [(In vitro P-gp efflux ratio) – 1/P-gp protein expression levels in P-gp–transfected LLC-PK1 cells]. Therefore, the species differences in the $K_{p,brain}$ between cynomolgus monkeys and mice are described as follows:
Using eqs. 2 and 5, the brain-to-plasma concentration ratios in cynomolgus monkeys and mice are described as follows:

\[
\frac{K_{p,\text{brain}}}{K_{p,\text{plasma}}} = \frac{f_{u,\text{brain.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \times \frac{f_{u,\text{plasma.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \tag{7}
\]

And using eqs. 2 and 5, the brain-to-plasma concentration ratios in rats and mice are described as follows:

\[
\frac{K_{p,\text{brain}}}{K_{p,\text{plasma}}} = \frac{f_{u,\text{brain.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \times \frac{f_{u,\text{plasma.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \tag{8}
\]

Therefore, the species differences in the ratio of the brain-to-plasma concentration in cynomolgus monkeys and mice are described as follows:

\[
\frac{K_{p,\text{brain}}}{K_{p,\text{plasma}}} = \frac{f_{u,\text{brain.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \times \frac{f_{u,\text{plasma.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \tag{9}
\]

In the present study, the contributions of individual parameters to the species differences in the ratio of the brain-to-plasma concentration in cynomolgus monkeys and mice were described as the Impact on the ratio of the brain-to-plasma concentration in cynomolgus monkeys and the Impact on the ratio of the brain-to-plasma concentration in mice, respectively. To quantitatively evaluate the contributions of each parameter, the Impact on the ratio of the brain-to-plasma concentration in cynomolgus monkeys and the Impact on the ratio of the brain-to-plasma concentration in mice were calculated using the following equations:

\[
\text{Impact on } \frac{K_{p,\text{monkey}}}{K_{p,\text{plasma}}} = \frac{f_{u,\text{plasma.mono}}} {f_{u,\text{brain.mono}}} \times \frac{f_{u,\text{brain.mono}}} {1 + TA_{\text{int.p-gp}} \times PL_{p-gp,\text{vivo}}} \tag{10}
\]

\[
\text{Impact on } \frac{K_{p,\text{mouse}}}{K_{p,\text{plasma}}} = \frac{K_{p,\text{monkey}}} {K_{p,\text{mouse}}} = \frac{K_{p,\text{monkey}}}{K_{p,\text{plasma}}} \times \frac{f_{u,\text{brain.mono}}} {f_{u,\text{plasma.mono}}} \tag{11}
\]

where the K_{p,\text{monkey}}/K_{p,\text{plasma}} consists of three cynomolgus monkey parameters and one mouse parameter. Briefly, the mouse data were used for either one of the P-gp-transfected cell lines or the mouse cell lines, and the cynomolgus monkey data were used for the other P-gp-transfected cell lines. The ordinate represents the amount of each compound that was transported from the donor side (500 μl) to the acceptor side (500 μl). Each point represents the mean ± S.D. (n = 3). 

\[\text{Transport (mol/patch, well)}\]

\[\text{Time (min)}\]

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasma Concentration</th>
<th>K_{p,\text{brain}}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>2 h</td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>mgl brain</td>
</tr>
<tr>
<td>Indinavir</td>
<td>BLQ</td>
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<tr>
<td>Quinidine</td>
<td>BLQ</td>
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<td>Loperamide</td>
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<td>Paclitaxel</td>
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</tr>
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<td>Diazepam</td>
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<td>0.0859</td>
</tr>
<tr>
<td>Verapamil</td>
<td>BLQ</td>
<td>0.601</td>
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</tbody>
</table>

BLQ, below the limit of quantification; Pre, just prior to the administration of the compounds.

Fig. 1. Transepithelial transport of five P-gp substrates and one nonsubstrate across cynomolgus monkey P-gp–transfected and parental LLC-PK1 cell monolayers. Six compounds were tested at 0.5 μM (indinavir (A), quinidine (B), loperamide (C), nonsubstrate diazepam (E), and verapamil (F), or 1 μM paclitaxel (D)) concentrations. The ordinate represents the amounts of each compound that were transported from the donor side (500 μl) to the acceptor side (500 μl). Each point represents the mean ± S.D. (n = 3).
on the basis of the results shown in Fig. 1, the apical-to-basal (A to B) transport rate, the basal-to-apical (B to A) transport rate, the flux ratio, and the in vitro P-gp efflux ratio across LLC-PK1/cynomolgus monkey P-gp transfected LLC-PK1 cell monolayers were calculated as described in Materials and Methods. For the quantifications of P-gp expression, cynomolgus monkey P-gp–transfected LLC-PK1 cells were cultured under the same conditions that were used for the transcellular transport experiments, and the expression levels in the transfected cell monolayers were determined using LC-MS/MS-based quantification (triplicate experiments). After the constant infusion of each microvessels of each cynomolgus monkey were determined using LC-MS/MS. Ratios and the P-gp protein expression levels using eq. 2. Each value represents the mean ± S.E.M. The S.E.M. was calculated according to the law of propagation of error.

### Table 2: Reconstruction of In Vivo P-gp Function (Kₚ,brain Ratio) at the Cynomolgus Monkey BBB

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transport Rate</th>
<th>P-gp Transfected LLC-PK1</th>
<th>Cynomolgus Monkey P-gp</th>
<th>Reconstructed Kₚ,brain</th>
<th>Reconstructed Kₚ,brain</th>
<th>Reconstructed Kₚ,brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A to B</td>
<td>B to A</td>
<td>A to B</td>
<td>B to A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indinavir</td>
<td>2.8 ± 0.05</td>
<td>298 ± 41</td>
<td>2.37 ± 0.15</td>
<td>3.38 ± 0.11</td>
<td>3.8 ± 0.15</td>
<td>3.8 ± 0.15</td>
</tr>
<tr>
<td>Quinidine</td>
<td>3.2 ± 0.04</td>
<td>744 ± 44</td>
<td>2.21 ± 0.24</td>
<td>7.28 ± 0.44</td>
<td>2.21 ± 0.24</td>
<td>2.21 ± 0.24</td>
</tr>
<tr>
<td>Loperamide</td>
<td>7.9 ± 0.09</td>
<td>603 ± 46</td>
<td>0.23 ± 0.08</td>
<td>3.0 ± 0.28</td>
<td>0.23 ± 0.08</td>
<td>0.23 ± 0.08</td>
</tr>
<tr>
<td>Verapamil</td>
<td>6.5 ± 0.08</td>
<td>989 ± 54</td>
<td>4.20 ± 0.03</td>
<td>9.3 ± 0.40</td>
<td>4.20 ± 0.03</td>
<td>4.20 ± 0.03</td>
</tr>
<tr>
<td>Diazepam</td>
<td>1.3 ± 0.07</td>
<td>246 ± 32</td>
<td>1.51 ± 0.05</td>
<td>9.2 ± 0.37</td>
<td>1.51 ± 0.05</td>
<td>1.51 ± 0.05</td>
</tr>
</tbody>
</table>

### Results

**Determination of the Steady-State Brain-to-Plasma Concentration Ratios of Five P-gp Substrates and One Nonsubstrate in Cynomolgus Monkeys.** Indinavir, quinidine, loperamide, paclitaxel, verapamil, and a nonsubstrate, diazepam, were administered to male adult cynomolgus monogolus monkeys by continuous intravenous infusion, and the Kₑ,brain values were determined at steady-state (3-hour) plasma concentrations (Table 1). The plasma concentrations of the six compounds at 3 hours ranged from 0.0331 to 0.514 μM, which were lower than the reported Kₑ,brain values for P-gp that were determined using the ATPase assay (Adachi et al., 2001). The Kₑ,brain values of the six compounds in cynomolgus monkeys varied by 15-fold (Table 1).

**Reconstruction of In Vivo P-gp Function (Kₑ,brain Ratio) at the Cynomolgus Monkey BBB.** The Kₑ,brain ratio is defined as the ratio of Kₑ,brain in P-gp knockout animals to that in wild-type animals and is a parameter that describes in vivo P-gp function at the BBB. The reconstruction of the Kₑ,brain ratio from the in vitro experiments has been previously demonstrated in mice (Uchida et al., 2011a). To demonstrate the reconstruction theory in cynomolgus monkeys as well as mice, we reconstructed the Kₑ,brain ratios for the six model compounds on the basis of the in vitro transport activities and protein expression levels of cynomolgus monkey P-gp according to the theory that was previously demonstrated in mice (eq. 2) as follows:

In the transcellular transport experiments using cynomolgus monkey P-gp–transfected LLC-PK1 cell monolayers and the parental LLC-PK1 cell monolayers shown in Fig. 1, the in vitro P-gp efflux ratio (a parameter reflecting the P-gp–specific transport activities of the test compounds) was determined and was found to range from 0.984 (diazepam) to 8.00 (loperamide) for the six compounds investigated (Table 2).

The protein expression levels of cynomolgus monkey P-gp were determined using QTAP. The levels were 2.31 fmol/μg protein of whole-cell lysate in cynomolgus monkey P-gp–transfected LLC-PK1 cell monolayers and ranged from 5.05 to 7.07 fmol/μg protein of whole-tissue lysate in the isolated brain microvessels among the six cynomolgus monkeys administered the six compounds respectively, including data taken from the literature (Ito et al., 2011) (Table 2).

Using these data, the Kₑ,brain ratios for the six compounds were reconstructed using eq. 2. The reconstruction of the Kₑ,brain ratio for each compound was performed using P-gp protein expression levels in the cynomolgus monkey that had been administered the corresponding compound. The reconstructed Kₑ,brain ratios of the six compounds ranged from 0.951 (diazepam) to 16.3 (loperamide) (Table 2).
in vivo compounds in cynomolgus monkeys were reconstructed as the ex vivo counterparts of the estimated in vivo values for all six compounds (Fig. 2B).

9 (reconstructed cynomolgus monkey values to the mouse values using eqs. 7 and 8) in vitro experimental data, we reconstructed the ratios of the unbound fractions in the brain, we cited the observed unbound fractions in vitro and the unbound fractions using eq. 4. The reconstructed K_{p,uu,brain} values were within a 3-fold range of the observed values for all six compounds (Fig. 2A).

Reconstruction of the K_{p,brain} of the Five P-gp Substrates and One Nonsubstrate in Cynomolgus Monkeys. Using eq. 5, the K_{p,brain} values for the six compounds in cynomolgus monkeys were reconstructed as the reciprocals of the reconstructed K_{p,uu,brain} values (Table 4). The in vivo K_{p,brain} values were estimated using the observed K_{p,brain} fractions in vivo and the unbound fractions using eq. 4. The reconstructed K_{p,brain} values were within a 3-fold range of the estimated in vivo values for all six compounds (Fig. 2B).

Reconstruction of Species Differences in the K_{p,brain} and K_{p,uu,brain} between Cynomolgus Monkeys and Mice. To clarify the species differences in drug distribution in the brain, we cited the observed K_{p,brain} and K_{p,uu,brain} values in mice reported in Uchida et al. (2011a), and divided the observed values in cynomolgus monkeys by those in mice (Fig. 3). The K_{p,brain} values indicated that the differences between species (observed K_{p,monkey}/K_{p,mouse}) were within a 0.657- to 10.9-fold range for the six compounds, with loperamide displaying the greatest difference (10.9-fold), followed by verapamil (5.22-fold). The species differences in the K_{p,uu,brain} values of each of the six compounds (estimated in vivo K_{p,uu,brain}/K_{p,uu,mouse}) were within a 0.510- to 2.44-fold range.

It is important for establishing the theory to quantitatively predict and overcome the species differences in brain drug distribution. To elucidate whether the species differences in K_{p,brain} and K_{p,uu,brain} values could be reconstructed from the in vitro experimental data, we reconstructed the ratios of the cynomolgus monkey values to the mouse values using eqs. 7 and 9 (reconstructed K_{p,monkey}/K_{p,mouse} and reconstructed K_{p,uu,monkey}/K_{p,uu,mouse}) and then compared these ratios with the observed ratios (Fig. 3). For both the K_{p,brain} and K_{p,uu,brain} values, the reconstructed ratios were within a 3-fold range of the observed ratios, although the reconstructed ratios for indinavir were 3.13-fold higher than the observed ratios.

Quantitative Evaluation of the Contributions of Individual Parameters to Species Differences in the K_{p,brain} and K_{p,uu,brain} between Cynomolgus Monkeys and Mice. To understand the mechanisms underlying the remarkable species differences in the K_{p,brain} for loperamide and verapamil, we estimated the contributions of four parameters to the species differences in the K_{p,brain} by calculating the “Impact on K_{p,monkey}/K_{p,mouse}” using eq. 10.
TABLE 4
Reconstructed and estimated in vivo $K_{p,uu,brain}$ values of the six compounds in cynomolgus monkeys

The reconstructed $K_{p,uu,brain}$ values were calculated as the reciprocals of the reconstructed $K_{p,brain}$ values (see Table 2) using eq. 5. The in vivo $K_{p,uu,brain}$ values were estimated from the $f_{a,plasma}$ and $f_{a,brain}$ (homogenate + pH partition model) values and the observed $K_{p,brain}$ values in cynomolgus monkeys (see Tables 1 and 3) using eq. 4. Each value represents the mean ± S.E.M. The S.E.M. was calculated according to the law of propagation of error.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reconstructed $K_{p,uu,brain}$ Value</th>
<th>Estimated In Vivo $K_{p,uu,brain}$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indinavir</td>
<td>0.179 ± 0.021</td>
<td>0.0607 ± 0.0039</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.0938 ± 0.0166</td>
<td>0.0744 ± 0.0040</td>
</tr>
<tr>
<td>Loperamide</td>
<td>0.0613 ± 0.0099</td>
<td>0.0400 ± 0.0035</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.130 ± 0.022</td>
<td>0.113 ± 0.008</td>
</tr>
<tr>
<td>Diazepam</td>
<td>1.05 ± 0.20</td>
<td>0.877 ± 0.036</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.195 ± 0.022</td>
<td>0.154 ± 0.010</td>
</tr>
</tbody>
</table>

(Tables 5). For loperamide, the species differences in $f_{a,plasma}$ and $P_l$-p-gp,vivo were estimated to contribute to the 3.37- and 2.68-fold higher $K_{p,monkey}$ than $K_{p,mouse}$, respectively, which were greater contributions than those of TAint,p-gp (0.550) and $f_{a,brain}$ (1.33). For verapamil, the species differences in $P_l$-p-gp,vivo $f_{a,plasma}$ and $f_{a,brain}$ were estimated to contribute to the 2.38-, 1.92-, and 1.77-fold higher $K_{p,monkey}$ than $K_{p,mouse}$, respectively.

For all six compounds, the species differences in $P_l$-p-gp,vivo and TAint,p-gp were estimated to contribute to the 2.16- to 2.68-fold (with the exception of diazepam) and 0.550- to 1.22-fold species differences in both the $K_{p,brain}$ and $K_{p,uu,brain}$, respectively (Table 5). The species differences in $f_{a,plasma}$ and $f_{a,brain}$ only affected the $K_{p,brain}$, not the $K_{p,uu,brain}$, and were estimated to contribute to the species differences in the $K_{p,brain}$ of 0.369- to 11.1-fold and 0.313- to 1.87-fold, respectively, for the six compounds.

Discussion

The present study is the first to experimentally demonstrate that the $K_{p,brain}$ values of P-gp substrates and nonsubstrate can be reconstructed in nonhuman primates. P-gp substrates and nonsubstrate can be reconstructed in nonhuman primates. P-gp substrates and nonsubstrate can be reconstructed in nonhuman primates.

In vivo P-gp transport function at the BBB is defined as the $K_{p,brain}$ ratio, which is the ratio of $K_{p,brain}$ value in P-gp knockout animals to that in wild-type animals. Because a P-gp knockout monkey does not exist, we were unable to determine the observed value of $K_{p,brain}$ ratio in monkeys, thereby making it impossible to directly validate the IVIVR of $K_{p,brain}$ ratio in monkeys. Equation 3 indicates that the accuracy of reconstruction of $K_{p,brain}$ value is directly influenced by that of $K_{p,brain}$ ratio. Therefore, the successful reconstruction of $K_{p,brain}$ value in this study has indirectly demonstrated that the $K_{p,brain}$ ratio can also be precisely reconstructed from in vitro P-gp transport activity and P-gp protein expression levels in monkeys. P-gp protein expression levels in brain microvessels only differ by 1.29-fold from those in humans (Ito et al., 2011; Uchida et al., 2011b), and a good agreement between monkey P-gp and human P-gp has been also reported in the in vitro transport activities for a variety of substrates (Takeuchi et al., 2006). Therefore, the present demonstration of reconstruction theory suggests that our established PPx-based reconstruction would be useful in clarifying in vivo P-gp function at the human BBB.

Significant species differences in $K_{p,brain}$ values were observed between monkeys and mice, with a maximum difference of 10.9-fold among six compounds (Fig. 3). Syvanen et al. (2009) also reported remarkable species differences in the $K_{p,brain}$ values of P-gp substrates between humans and rats (e.g., an 8.6-fold difference for $[11C]GR205171). On the basis of these results, it is clear that the $K_{p,brain}$ values measured in rodent experiments cannot reliably predict drug distributions in human brain. Several studies have suggested the usefulness of in vitro P-gp–transfected cells for predictions of in vivo P-gp functions at the BBB and drug distribution in brain (Adachi et al., 2001; Feng et al., 2008). However, it is challenging to make accurate predictions on the basis of in vitro experiments because the extent to which the transport functions and protein expression levels of P-gp differ between in vivo BBB and in vitro transfected cells is unclear. In this study, using monkeys that are similar to humans in terms of drug distribution in brain, we demonstrated that the $K_{p,brain}$ and $K_{p,uu,brain}$ values can be predicted with ±3-fold accuracy from in vitro experiments on the basis of the in vivo differences in protein expression levels of P-gp (Fig. 2).
Furthermore, we demonstrated that the species differences in $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ values can also be predicted from in vitro studies with &lt;= 3-fold accuracy (Fig. 3). Therefore, our established PPIX-based reconstruction overcomes the species differences in drug distribution in brain and provides a useful method to rationally predict drug distribution in human brain from in vitro experiments.

One of the advantages of $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ reconstructions on the basis of several factors is that the contributions of individual factors can be quantitatively evaluated for changes in $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ values. As shown in Fig. 3, remarkable species differences in $K_{p,\text{brain}}$ values were observed for loperamide and verapamil between monkeys and mice. Table 5 summarizes the contributions of four factors [$\text{BBB P-gp protein expression levels (PL}_{p,\text{gg, vivo}}, \text{intrinsic transport activity per P-gp molecule (TA}_{\text{int,p-gp}}, f_{u,\text{plasma}}, \text{and } f_{u,\text{brain}})$] to the species differences in $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ Values of six compounds. For loperamide, the species differences in $f_{u,\text{plasma}}$ and $\text{PL}_{p,\text{gg, vivo}}$ were 3.37- and 2.79-fold, respectively, and contributed to 3.37- and 2.68-fold species differences in $K_{p,\text{brain}}$ values, respectively. Smaller contributions were observed for the other two factors. These results suggest that major causes of the species differences in $K_{p,\text{brain}}$ values of loperamide are differences in $f_{u,\text{plasma}}$ and $\text{PL}_{p,\text{gg, vivo}}$ between monkeys and mice. For verapamil, 2.71- 1.92, and 1.77-fold species differences in $\text{PL}_{p,\text{gg, vivo}}, f_{u,\text{plasma}},$ and $f_{u,\text{brain}}$ were observed, respectively, and these differences contributed to 2.38-, 1.92-, and 1.77-fold greater $K_{p,\text{brain}}$ values in monkeys than in mice, respectively. These data suggest that the 5.22-fold greater observed $K_{p,\text{brain}}$ values for verapamil in monkeys are caused by the species differences in these three factors.

Among the four factors, the species differences in $f_{u,\text{plasma}}$ showed the largest variation among six compounds, ranging from 0.369 (quindine) to 11.1-fold (indinavir) differences between monkeys and mice (Table 5). The Impact on $K_{p,\text{monkey}}/K_{p,\text{mouse}}$ of $f_{u,\text{plasma}}$ also varied from 0.369 (quinidine) to 11.1 (indinavir), which was the largest variation among the four factors, suggesting that $f_{u,\text{plasma}}$ most significantly contributes to species variations in $K_{p,\text{brain}}$ values. Several studies using a variety of compounds have indicated that there are remarkable species differences in $f_{u,\text{plasma}}$ but no large differences in $f_{u,\text{brain}}$ (Fuse et al., 1998; Kratochwil et al., 2004; Di et al., 2011). Table 5 shows that $f_{u,\text{brain}}$ did not affect the species differences in $K_{p,\text{brain}}$ values of six compounds to as great an extent as $f_{u,\text{plasma}}$. Therefore, unlike $f_{u,\text{plasma}}, f_{u,\text{brain}}$ would not contribute substantially to species variations in $K_{p,\text{brain}}$ values.

The $K_{p,\text{uu,brain}}$ value is independent of $f_{u,\text{plasma}}$ and $f_{u,\text{brain}}$ values, as shown in eq. 5 and Table 5, and consequently species differences in $K_{p,\text{uu,brain}}$ values were smaller than those in $K_{p,\text{brain}}$ values (Fig. 3). As shown in eqs. 2 and 5, the $K_{p,\text{uu,brain}}$ value is affected only by $\text{PL}_{p,\text{gg, vivo}}$ and $\text{TA}_{\text{int,p-gp}}$, $\text{PL}_{p,\text{gg, vivo}}$ differed by 2.00- to 2.79-fold between monkeys and mice, and $\text{TA}_{\text{int,p-gp}}$ did not differ so much (Table 5). As a result, the Impact on $K_{p,\text{uu,monkey}}/K_{p,\text{uu,mouse}}$ of $\text{PL}_{p,\text{gg, vivo}}$ ranged from 2.16- to 2.68-fold (with the exception of dazepam, which is not a P-gp substrate) and was greater than that of $\text{TA}_{\text{int,p-gp}}$ (Table 5). These data suggest that the $\text{PL}_{p,\text{gg, vivo}}$ is

### Table 5

Impact of four parameters on species differences in the $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ Values between cynomolgus monkeys and mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Impact on $K_{p,\text{monkey}}/K_{p,\text{mouse}}$</th>
<th>$f_{u,\text{plasma}}$</th>
<th>$f_{u,\text{brain}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indinavir</td>
<td>Monkey/Mouse</td>
<td>2.16</td>
<td>1.11</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Monkey/Mouse</td>
<td>2.33</td>
<td>1.37</td>
</tr>
<tr>
<td>Loperamide</td>
<td>Monkey/Mouse</td>
<td>2.68</td>
<td>1.37</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Monkey/Mouse</td>
<td>2.48</td>
<td>1.37</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Monkey/Mouse</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Monkey/Mouse</td>
<td>2.38</td>
<td>1.37</td>
</tr>
</tbody>
</table>

TABLE 5

Impact of four parameters on species differences in the $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ Values between cynomolgus monkeys and mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Impact on $K_{p,\text{monkey}}/K_{p,\text{mouse}}$</th>
<th>$f_{u,\text{plasma}}$</th>
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</thead>
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<tr>
<td>Indinavir</td>
<td>Monkey/Mouse</td>
<td>2.16</td>
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<td>Diazepam</td>
<td>Monkey/Mouse</td>
<td>1</td>
<td>1</td>
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<td>Verapamil</td>
<td>Monkey/Mouse</td>
<td>2.38</td>
<td>1.37</td>
</tr>
</tbody>
</table>
a major cause of the species differences in $K_{puu,brain}$ values. The species differences in P-gp protein expression levels at the BBB are 2- to 3-fold between humans and rodents, as is the case for monkeys and mice (Kamiie et al., 2008; Ito et al., 2011; Uchida et al., 2011b; Hoshi et al., 2013). Therefore, the differences in the $K_{puu,brain}$ values of P-gp substrates between humans and rodents could typically range from approximately 2- to 3-fold, in accordance with the differences in protein expression levels. From this consideration, it is suggested that the $K_{puu,brain}$ values of P-gp substrates differ between humans and rodents but do not remarkably differ when compared with the species differences in $K_{p,brain}$ values. Therefore, the measurement of $K_{puu,brain}$ value in rodents during drug development would be useful in understanding drug distribution in human brain.

The reconstructed brain distributions of six compounds in monkeys were within a 3-fold range of the observed distributions but were not completely identical (Fig. 2). One possible explanation is that the $f_{u,brain}$ used for the reconstruction was not identical to that in vivo. Brain slice method can provide $f_{u,brain}$ values that are more relevant to in vivo condition (Kakee et al., 1996; Ooie et al., 1997; Friden et al., 2007). However, frozen monkey brains were used in this study to mimic the way of IVIVR in humans, for which the brain is usually obtained after a frozen state. In frozen brain, the cells may be partially ruptured, and the $f_{u,brain}$ may not be accurately determined using the brain slice method. Therefore, we used the homogenate method with a pH partition method alone (Friden et al., 2011). However, this method does not take into account the involvement of active transport on the cell membrane of brain parenchyma, resulting in the possibility that the determined $f_{u,brain}$ differs from the true in vivo value. Another possible explanation is that only one monkey was studied for each compound to determine the observed $K_{puu,brain}$ values. The variability of observed values could not be considered owing to limited resources. Increasing the number of monkeys for each compound would raise the accuracy for the validation of the present IVIVR.

In conclusion, using cytomolgus monkeys as a robust human model, this study experimentally demonstrated that the $K_{p,brain}$ and $K_{puu,brain}$ values of P-gp substrates and non-substrate can be reconstructed by integrating in vitro P-gp transport activity, P-gp protein expression levels, and the unbound fractions in plasma and brain on the basis of BBB PPFx. These results also demonstrate that in vivo P-gp transport function at the BBB can be reconstructed on the basis of in vitro P-gp transport activity and P-gp protein expression levels. These demonstrations illustrate the value of our established PPFx-based reconstruction model for clarifying in vivo function at the human BBB and predicting brain drug distribution in humans. Because not only P-gp but also BCRP at the BBB limit brain distributions of a number of drugs, further study would be needed in future to demonstrate that in vivo function of BCRP can be reconstructed from in vitro for the prediction of brain distribution for more drugs.

Acknowledgments

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