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

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Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; f_u,brain, unbound fraction in brain; f_u,plasma, unbound fraction in plasma; IVIVE, in-vitro-to-in-vivo extrapolation; IVIVR, in-vitro-to-in-vivo reconstruction; K_p,brain, brain-to-plasma concentration ratio; K_p,uu,brain, unbound brain-to-plasma concentration ratio; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MDR1, multidrug resistance protein 1; PL_p-gp,vivo, protein expression level of P-gp in isolated brain microvessels; PET, positron emission tomography; P-gp, P-glycoprotein; PPx, pharmacoproteomics; QTAP, quantitative targeted absolute proteomics; S.D., standard deviation; S.E.M., standard error of the mean; SRM/MRM, selected/multiple reaction monitoring; TA_int,p-gp, intrinsic transport activity per P-gp molecule.

Recommended section: Metabolism, Transport, and Pharmacogenomics
Abstract (244 words / Max 250 words)

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/MDR1) 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 (K_p 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 K_p brain were within a three-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 the species differences in K_p brain between monkeys and mice. Unbound brain-to-plasma concentration ratios (K_p,uu,brain) were reconstructed as the reciprocal of the reconstructed in-vivo P-gp functions, and the reconstructed K_p,uu,brain were within a three-fold range of in-vivo values, which were estimated from observed K_p brain and unbound fractions. This study experimentally demonstrates that brain distributions of P-gp substrates and non-substrate can be reconstructed based on 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 to the number of drug candidates that progress from pre-clinical to clinical trials. The proportion was just 8% for central nervous system (CNS)-acting drugs during the period from 1991 to 2000 (Kola and Landis, 2004), and also no greater during the period from 2000 to 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 (Pardridge, 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’s necessary to determine quantitative and accurate prediction of drug distribution in human brain during the pre-clinical 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-gp/MDR1/mdr1a/ABCB1 and BCRP/ABCG2 are major gatekeepers for many drugs (Kusuhara and Sugiyama, 2009; Uchida et al., 2011b). Therefore, it’s 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 based on 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 using LC-MS/MS, termed “quantitative targeted absolute proteomics (QTAP)” (Kamiie et al.,
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), which is an integrated scientific field of proteomics and pharmacokinetics/pharmacodynamics/toxicokinetics/toxicodynamics to quantitatively understand drug ADME, pharmacological effect and toxicity (Uchida et al., 2014).

However, brain distributions of P-gp substrates, such as $[^{18}\text{F}]$altanserin and $[^{11}\text{C}]$GR205171, significantly differ between humans and rodents (Syvanen et al., 2009). Therefore, it’s debatable whether the demonstration of in-vitro-to-in-vivo reconstruction 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’s also important to overcome species difference in brain drug distribution. Brain-to-plasma concentration ratios ($K_p\text{ brain}$) of P-gp substrate verapamil and PF-00905556 in monkey are significantly 10.8- and 12.2-fold greater than those in mouse and rat, respectively (Hendrikse et al., 1998; Kpakima et al., 2006; Syvanen et al., 2009). Between monkey and rodents, the protein expression levels of P-gp/mdr1a at the BBB differ only by 3-4-fold (Kamiie et al., 2008; Ito et al., 2011; Hoshi et al., 2013). Therefore, the remarkable species differences in $K_p\text{ brain}$ cannot be completely explained by the differences in protein expression levels. It’s necessary to consider the species differences not only in protein expression levels but also in other factors such as intrinsic transport activity and unbound
fractions in plasma and brain. Although we have already succeeded the prediction of $K_{p,\text{brain}}$ by integrating all these factors in mouse model (Uchida et al., 2011a), the further validation in monkey would dramatically increase the reliability of prediction in humans.

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 ($K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$) of six model compounds including five P-gp substrates and one non-substrate in cynomolgus monkeys based on the theory that was previously established in a mouse model, and compared 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 quinidine were purchased from Sigma Chemical Co. (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, 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 (y) and 2 months (m) with a body weight (BW) of 3.6 kg was treated with indinavir; 2) aged 4 y 1 m with a BW of 2.6 kg was treated with quinidine; 3) aged 6 y 4 m with a BW of 5.4 kg was treated with loperamide; 4) aged 5 y 4 m with a BW of 3.4 kg was treated with paclitaxel; 5) aged 4 y 3 m with a BW of 3.15 kg was treated with diazepam; and 6) aged 4 y 1 m with a BW of 3.2 kg 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. (Ibaragi, 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 y 1 m with a BW of 3.3 kg, 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. (Ibaragi, Japan). 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 h without anesthesia. With the exception of loperamide and paclitaxel, intravenous bolus injections of the test compounds were performed immediately prior to the constant infusion to quickly reach the steady-state plasma concentration; indinavir, quinidine, diazepam, and verapamil were intravenously infused for 3 h at dose rates of 0.61, 0.21, 0.070, and 0.40 mg/h/kg, 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 h at dose rates of 0.20 and 0.94 mg/h/kg, respectively, without intravenous bolus injections. One cynomolgus monkey was used for each compound. Prior to administration and at 2, 2.5, and 3 h after administration, blood samples were collected using syringes containing EDTA-2K without anesthesia from the cephalic or saphenous vein on the opposite side of that used for administration. The blood samples were immediately centrifuged at 4°C and 3500 x g for 10 min to obtain plasma. The plasma samples were stored at -80°C prior to LC-MS/MS analyses. Immediately after the blood sampling at 3 h, 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 two-fold volume of phosphate-buffered saline 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 960 x g for 10 min and subjected to LC-MS/MS analyses. Based on pharmacokinetic concepts, the brain-to-plasma concentration ratios (\(K_{p \text{ brain}}\)) at 3 h were 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 x 10^6 cells/insert (0.484 x 10^6 cells/cm^2) on porous (1.0 μm) polyethylene terephthalate membrane filters (cell culture inserts for 24-well plates; BD Biosciences, Franklin Lakes, NJ) that had been coated with BD Matrigel™ Basement Membrane Matrix (BD Biosciences). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, supplemented with fresh culture medium on the second day, and used for the experiments on the fourth day after seeding. Hanks' balanced salt solution (HBSS) 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 D-glucose, 10 mM HEPES, pH 7.2-7.4) was named 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 h, 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 min) between sampling times (10, 20, and 30 min 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, the buffer in the insert was completely removed, 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 min), 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-MS/MS 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 (fmol/min/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 $2.081 \times 10^6$ cells/insert ($0.484 \times 10^6$ cells/cm$^2$), 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 were harvested from six inserts using 1 mL/insert of ice-cold PBS(-) by scraping and centrifuged at 4°C and 230 x g for 5 min. The cell pellets were dissolved with 400 μL of TS buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4) and suspended well using a 1.0 mL syringe with a 27G x 1/2” needle 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-and-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 micro-equilibrium dialysis device (HTD 96b; HTDialysis, Gales Ferry, CT), respectively, with HTD 96a/b Dialysis Membrane Strips (MWCO 12-14K; HTDialysis). The wells were sealed and rotated in a 10% CO₂ incubator at 37°C and 80 rpm with a multi-shaker (TOKYO RIKAKIKAI Co. Ltd., Tokyo, Japan). After a 6 h 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-and-fifty microliters of acetonitrile-containing buspirone (internal standard) was added (205 µL in total) and vortexed. The samples were centrifuged and filtrated at 4°C and 960 x g for 10 min and subjected to LC-MS/MS analyses. The unbound fractions of the test compounds in the cynomolgus monkey plasma were calculated based on 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 MWCO 14000 were shaken in distilled water for 30 min, washed with extracellular fluid (ECF) buffer (122 mM NaCl, 3 mM KCl, 0.4 mM K2HPO4, 25 mM NaHCO3, 1.4 mM CaCl2, 1.2 mM MgSO4, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), and conditioned in ECF buffer overnight. The cerebrum was diluted four-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 min, 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 EC-1 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 h with 300 rpm shaking. After 6 h, the brain homogenates and dialysate samples were collected from the apparatus. One-hundred-and-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 min, the samples were centrifuged at 4°C and 17,360 x g for 5 min. Then, 180 μL of the supernatant was evaporated by centrifugation under vacuum. The residue was then reconstituted in 0.1% aqueous formic acid and centrifuged at 4°C and 17,360 x g for 5 min. The supernatants were
subjected to LC-MS/MS analyses. The following equation was used to calculate the unbound fractions in the brain ($f_{u,\text{brain}}$), where $D$ represents the fold dilution of the brain tissue and $f_{u,\text{measured}}$ is the ratio of concentrations determined from the dialysate and brain homogenate samples:

$$f_{u,\text{brain}} = \frac{1}{D} \cdot \frac{1}{ \left( \frac{1}{f_{u,\text{measured}}} - 1 \right) + 1/D}$$

(1)

According to the pH partition model reported in Friden et al. (2011), $f_{u,\text{brain}}$ values that were more relevant to the in-vivo condition were calculated using the values of $f_{u,\text{brain}}$ determined using the homogenate method above (eq. 1) and the reported pKa values of the test compounds (see the legend for Table 3).

**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 (HPLC) system (Agilent Technologies, Santa Clara, CA). The samples were injected onto either an Agilent XDB-C18 column ($2.1 \times 150$ mm, $5 \, \mu\text{m}$) or a Shiseido Capcell pak UG120 C18 column ($2.0 \times 150$ mm, $5 \, \mu\text{m}$). 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 electro-spray ionization in selected/multiple reaction monitoring (SRM/MRM) mode. SRM/MRM 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.

**In-vitro-to-in-vivo reconstruction (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 non-substrate 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 $K_p^{\text{brain}}$ ratio, which is the ratio of $K_p^{\text{brain}}$ in P-gp knockout animals to that in wild-type animals. As previously described (Uchida et al., 2011a), the $K_p^{\text{brain}}$ ratio was reconstructed using the equation presented below (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.

$$
P - \text{gp protein expression}
K_p^{\text{brain}} \text{ ratio} = 1 + ((\text{in - vitro P - gp efflux ratio}) - 1) \times \frac{\text{levels in brain microvessels}}{\text{P - gp protein expression levels in P - gp - transfected cell monolayer}}
$$

(2)

The $K_p^{\text{brain}}$ in cynomolgus monkeys was reconstructed in eq. 3 using the reconstructed $K_p^{\text{brain}}$ ratio (eq. 2) and the unbound fractions in the plasma ($f_{u,\text{plasma}}$) and brain ($f_{u,\text{brain}}$), which were measured using the homogenate method and the pH partition model.

$$
K_p^{\text{brain}} = \frac{f_{u,\text{plasma}}}{f_{u,\text{brain}} \times K_p^{\text{brain}} \text{ ratio}}
$$

(3)

Furthermore, the $K_{p,uu,\text{brain}}$ is defined as follows:

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

(4)

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

$$
K_{p,uu,\text{brain}} = \frac{1}{K_p^{\text{brain}} \text{ ratio}}
$$

(5)

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

Quantitative evaluation of the effects of individual parameters on species differences in $K_p$ \text{brain} and $K_{p,uu,brain}$ between cynomolgus monkeys and mice: Using eq. 2 and eq. 3, the $K_p$ \text{brain} is described by four parameters, as follows:

$$K_p \text{brain} = \frac{f_{u,\text{plasma}}}{f_{u,\text{brain}}} x (1 + TA_{\text{int,p-gp}} x PL_{\text{p-gp,vivo}})$$

where PL_{p-gp,vivo} represents the protein expression levels of P-gp in isolated brain microvessels and TA_{int,p-gp} 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 $K_p$ \text{brain} between cynomolgus monkeys and mice are described as follows:

$$\frac{K_{p,\text{monkey}}}{K_{p,\text{mouse}}} = \frac{f_{u,\text{plasma,monkey}}}{f_{u,\text{plasma,mouse}}} x \frac{f_{u,\text{brain,monkey}}}{f_{u,\text{brain,mouse}}} x \frac{1 + TA_{\text{int,p-gp,mouse}} x PL_{\text{p-gp,vivo,mouse}}}{1 + TA_{\text{int,p-gp,monkey}} x PL_{\text{p-gp,vivo,monkey}}}$$

Using eq. 2 and eq. 5, the $K_{p,uu,brain}$ is described by two parameters, as follows:

$$K_{p,uu,brain} = \frac{1}{1 + TA_{\text{int,p-gp}} x PL_{\text{p-gp,vivo}}}$$

Therefore, the species differences in the $K_{p,uu,brain}$ between cynomolgus monkeys and mice are described as follows:
In the present study, the contributions of individual parameters to the species differences in the \( K_{p,\text{brain}} \) and \( K_{p,\text{brain},\text{uu}} \) between cynomolgus monkeys and mice were described as the “Impact on \( K_{p,\text{monkey}}/K_{p,\text{mouse}} \)” and the “Impact on \( K_{p,\text{uu,monkey}}/K_{p,\text{uu,mouse}} \)” respectively. To quantitatively evaluate the contributions of each parameter, the “Impact on \( K_{p,\text{monkey}}/K_{p,\text{mouse}} \)” and the “Impact on \( K_{p,\text{uu,monkey}}/K_{p,\text{uu,mouse}} \)” were calculated using the following equations:

\[
\text{Impact on } K_{p,\text{monkey}} / K_{p,\text{mouse}} = \frac{K_{p,\text{monkey}}}{K_{p,\text{mouse}}} \frac{K_{p,\text{monkey}}(\text{mouse})}{K_{p,\text{mouse}}} = \frac{K_{p,\text{monkey}}}{K_{p,\text{monkey}}(\text{mouse})} \frac{f_{u,\text{brain,monkey}}(\text{mouse})}{f_{u,\text{brain,monkey}}(\text{mouse})} \frac{1 + TA_{\text{int},\text{p-gp,monkey}}(\text{mouse})}{1 + TA_{\text{int},\text{p-gp,monkey}}(\text{mouse})} \frac{PL_{p-gp,vivo,\text{monkey}}}{PL_{p-gp,vivo,\text{monkey}}} \tag{10}
\]

\[
\text{Impact on } K_{p,\text{uu,monkey}} / K_{p,\text{uu,mouse}} = \frac{K_{p,\text{uu,monkey}}}{K_{p,\text{uu,mouse}}} \frac{K_{p,\text{uu,monkey}}(\text{mouse})}{K_{p,\text{uu,monkey}}(\text{mouse})} = \frac{K_{p,\text{uu,monkey}}}{K_{p,\text{uu,monkey}}(\text{mouse})} \frac{f_{u,\text{brain,monkey}}(\text{mouse})}{f_{u,\text{brain,monkey}}(\text{mouse})} \frac{1 + TA_{\text{int},\text{p-gp,monkey}}(\text{mouse})}{1 + TA_{\text{int},\text{p-gp,monkey}}(\text{mouse})} \frac{PL_{p-gp,vivo,\text{monkey}}}{PL_{p-gp,vivo,\text{monkey}}} \tag{11}
\]

where the \( K_{p,\text{monkey}}(\text{mouse}) \) consists of three cynomolgus monkey parameters and one mouse parameter. Briefly, the mouse data were used for either one of \( PL_{p-gp,vivo,\text{monkey}}(\text{mouse}) \), \( TA_{\text{int},\text{p-gp,monkey}}(\text{mouse}) \), \( f_{u,\text{plasma,monkey}}(\text{mouse}) \), or \( f_{u,\text{brain,monkey}}(\text{mouse}) \), which was a targeted parameter for the evaluation of contribution, and the cynomolgus monkey data were used for the other three parameters. In the same manner as the \( K_{p,\text{monkey}}(\text{mouse}) \), only one of the evaluated parameters was derived from the mouse data and the others were derived from the cynomolgus monkey data for the \( K_{p,\text{uu,monkey}}(\text{mouse}) \). For the cynomolgus monkey data, the
experimental values that were determined in the present study were used. For the mouse data, the values reported by Uchida et al. (2011a) were used.
Results

Determination of the steady-state brain-to-plasma concentration ratios ($K_{p,\,\text{brain}}$) of five P-gp substrates and one non-substrate in cynomolgus monkeys: Indinavir, quinidine, loperamide, paclitaxel, verapamil, and a non-substrate diazepam were administered to male adult cynomolgus monkeys by continuous intravenous infusion, and the $K_{p,\,\text{brain}}$ values were determined at steady-state (3 h) plasma concentrations (Table 1). The plasma concentrations of the six compounds at 3 h ranged from 0.0331 to 0.541 $\mu$M, which were lower than the reported $K_m$ values for P-gp that were determined using the ATPase assay (Adachi et al., 2001). The $K_{p,\,\text{brain}}$ values of the six compounds in cynomolgus monkeys varied by 15-fold (Table 1).

Reconstruction of in-vivo P-gp function ($K_{p,\,\text{brain\ ratio}}$) at the cynomolgus monkey BBB: The $K_{p,\,\text{brain}}$ ratio is defined as the ratio of $K_{p,\,\text{brain}}$ 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 $K_{p,\,\text{brain}}$ 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 $K_{p,\,\text{brain}}$ ratios for the six model compounds based on 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 quantitative targeted absolute proteomics (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 6 cynomolgus monkeys administered the 6 compounds respectively, including data taken from the literature (Ito et al., 2011) (Table 2).

Using these data, the $K_p$ brain ratios for the six compounds were reconstructed using eq. 2. The reconstruction of the $K_p$ 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 $K_p$ brain ratios of the six compounds ranged from 0.951 (diazepam) to 16.3 (loperamide) (Table 2).

**Reconstruction of the $K_p$ brain of the five P-gp substrates and one non-substrate in cynomolgus monkeys:** The $K_p$ brain values for the six compounds in cynomolgus monkeys were reconstructed using eq. 3 and the reconstructed $K_p$ brain ratios and the unbound fractions in cynomolgus monkey plasma and brain (Table 3). The unbound fractions in the brain were measured using the homogenate method, converted to values that were more relevant to the in-vivo condition in combination with the pH partition model, and used for the $K_p$ brain reconstructions. The reconstructed $K_p$ brain values were within a three-fold range of the observed values for all six compounds (Fig. 2A).

**Reconstruction of the $K_{p,uu,brain}$ of the five P-gp substrates and one non-substrate in cynomolgus monkeys:** Using eq. 5, the $K_{p,uu,brain}$ values for the six compounds in cynomolgus monkeys were reconstructed as the reciprocals of the reconstructed $K_p$ brain ratios (Table 4). The in-vivo $K_{p,uu,brain}$ values were estimated using the observed $K_p$ brain values in-vivo and the
unbound fractions using eq. 4. The reconstructed $K_{p,\text{uu,brain}}$ values were within a three-fold range of the estimated in-vivo values for all six compounds (Fig. 2B).

**Reconstruction of species differences in the $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ between cynomolgus monkeys and mice:** To clarify the species differences in drug distribution in the brain, we cited the observed $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ values in mice reported in Uchida et al. (2011a), and divided the observed values in cynomolgus monkeys by those in mice (Fig. 3). The $K_{p,\text{brain}}$ values indicated that the differences between species (observed $K_{p,\text{monkey}}/K_{p,\text{mouse}}$) were within a 0.657-10.9-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 $K_{p,\text{uu,brain}}$ values of each of the six compounds (Estimated in-vivo $K_{p,\text{uu,monkey}}/K_{p,\text{uu,mouse}}$) were within a 0.510-2.44-fold range.

It is important to establish the theory to quantitatively predict and overcome the species differences in brain drug distribution. To elucidate whether the species differences in $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ values could be reconstructed from the in-vitro experimental data, we reconstructed the ratios of the cynomolgus monkey values to the mouse values using eq. 7 and 9 (reconstructed $K_{p,\text{monkey}}/K_{p,\text{mouse}}$ and reconstructed $K_{p,\text{uu,monkey}}/K_{p,\text{uu,mouse}}$) and then compared these ratios with the observed ratios (Fig. 3). For both the $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ values, the reconstructed ratios were within a three-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 $K_{p,\text{brain}}$ and $K_{p,\text{uu,brain}}$ between cynomolgus monkeys and mice:** To understand the mechanisms underlying the remarkable species differences in the $K_{p,\text{brain}}$ 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 5). For loperamide, the species differences in $f_{u,plasma}$ and $PL_{p-gp,vivo}$ 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 $TA_{int,p-gp}$ (0.550) and $f_{u,brain}$ (1.33). For verapamil, the species differences in $PL_{p-gp,vivo}$, $f_{u,plasma}$, and $f_{u,brain}$ 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 $PL_{p-gp,vivo}$ and $TA_{int,p-gp}$ were estimated to contribute to the 2.16-2.68-fold (except for diazepam) and 0.550-1.22-fold species differences both in the $K_p$ brain and $K_{p,uu,brain}$, respectively (Table 5). The species differences in $f_{u,plasma}$ and $f_{u,brain}$ 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 0.369-11.1-fold and 0.313-1.87-fold, respectively, for the six compounds.
Discussion

The present study is the first to experimentally demonstrate that the \( K_{p,\text{brain}} \) values of P-gp substrates and non-substrate can be reconstructed in non-human primate cynomolgus monkeys, an animal that is similar to humans, by integrating in-vitro P-gp transport activity, P-gp protein expression levels, and the unbound fractions in plasma and brain based on eq. 2 and eq. 3. This study is also the first to experimentally demonstrate that the \( K_{p,\text{uu,brain}} \) values can be reconstructed by integrating in-vitro P-gp transport activity and P-gp protein expression levels based on eq. 2 and eq. 5.

In-vivo P-gp transport function at the BBB is defined as the \( K_{p,\text{brain}} \) ratio, which is the ratio of \( K_{p,\text{brain}} \) 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 \( K_{p,\text{brain}} \) ratio in monkeys, thereby making it impossible to directly validate the in-vitro-to-in-vivo reconstruction of \( K_{p,\text{brain}} \) ratio in monkeys. Eq. 3 indicates that the accuracy of reconstruction of \( K_{p,\text{brain}} \) value is directly influenced by that of \( K_{p,\text{brain}} \) ratio. Therefore, the successful reconstruction of \( K_{p,\text{brain}} \) value in this study has indirectly demonstrated that the \( K_{p,\text{brain}} \) 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 pharmacoproteomics (PPx)-based reconstruction would be useful in clarifying in-vivo P-gp function at the human BBB.

Significant species differences in \( K_{p,\text{brain}} \) 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 $K_p\text{brain}$ values of P-gp substrates between humans and rats (e.g., an 8.6-fold difference for $[^{11}\text{C}]\text{GR205171}$). Based on these results, it is clear that the $K_p\text{brain}$ 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 based on 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 $K_p\text{brain}$ and $K_{p,\text{uu,brain}}$ values can be predicted with $\pm$ three-fold accuracy from in-vitro experiments based on the in-vitro/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 $\pm$ three-fold accuracy (Fig. 3). Therefore, our established PPx-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 based on 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 (BBB P-gp protein expression levels ($PL_{p,\text{gp,vivo}}$), intrinsic transport activity per P-gp molecule ($TA_{\text{int,p-gp}}$), $f_u,\text{plasma}$, 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 $PL_{p,\text{gp,vivo}}$ 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 $PL_{p,\text{gp,vivo}}$ between monkeys and mice. For verapamil, 2.71-, 1.92-, and 1.77-fold species differences in $PL_{p,\text{gp,vivo}}$, $f_{u,\text{plasma}}$, 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- (quinidine) 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 the 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 eq. 2 and eq. 5, the $K_{p,\text{uu,brain}}$ value is affected only by $PL_{p,\text{gp,vivo}}$ and $TA_{\text{int,p-gp}}$. $PL_{p,\text{gp,vivo}}$ differed by 2.00- to 2.79-fold between monkeys and mice, and $TA_{\text{int,p-gp}}$ did not differ so much (Table 5). As a result, the “Impact on $K_{p,\text{uu,monkey}}/K_{p,\text{uu,mouse}}$” of $PL_{p,\text{gp,vivo}}$ ranged from 2.16- to 2.68-fold (with the exception of diazepam, which is not a P-gp substrate) and was greater than that of $TA_{\text{int,p-gp}}$ (Table 5).
These data suggest that the PL_{p-gp,vivo} is a major cause of the species differences in K_{p,uu,brain} values. The species differences in P-gp protein expression levels at the BBB are two- to three-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_{p,uu,brain} values of P-gp substrates between humans and rodents could typically range from approximately two- to three-fold, in accordance with the differences in protein expression levels. From this consideration, it is suggested that the K_{p,uu,brain} values of P-gp substrates differ between humans and rodents but do not remarkably differ when compared to the species differences in K_{p,brain} values. Therefore, the measurement of K_{p,uu,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 three-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 in-vitro-to-in-vivo reconstruction in humans, for which the brain is usually obtained in 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 model, which results in an f_u,brain value that is more relevant to in-vivo condition than that obtained using the homogenate 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_p brain values. The variability of observed values could not be
considered due to a limited resources. Increasing the number of monkey for each compound would raise the accuracy for the validation of the present in-vitro-to-in-vivo reconstruction.

In conclusion, using cynomolgus monkeys as a robust human model, this study experimentally demonstrated that the $K_p^{\text{brain}}$ and $K_{p,uu,\text{brain}}$ 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 based on BBB PPx. These results also demonstrate that in-vivo P-gp transport function at the BBB can be reconstructed based on in-vitro P-gp transport activity and P-gp protein expression levels. These demonstrations illustrate the value of our established PPx-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.
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Author Contributions

Participated in the research design: Uchida, Wakayama, Ohtsuki, Chiba, Ohe, Ishii, and Terasaki

Conducted the experiments: Uchida and Wakayama

Contributed new reagents or analytical tools: Uchida and Wakayama

Performed the data analyses: Uchida and Wakayama

Wrote or contributed to the writing of the manuscript: Uchida, Ohtsuki, and Terasaki
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Footnotes

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Regarding conflicts of interest, Tetsuya Terasaki and Sumio Ohtsuki are full professors at Tohoku University and Kumamoto University, respectively, and are also directors of Proteomedix Frontiers Co. Ltd. This study was not supported by Proteomedix Frontiers Co. Ltd., and their positions at Proteomedix Frontiers Co. Ltd. did not affect the design of the study, the collection of the data, the analysis or interpretation of the data, the decision to submit the manuscript for publication, or the writing of the manuscript and did not present any financial conflicts. The other authors declare that no competing interests exist.
Figure Legends

Figure 1: Transepithelial transport of five P-gp substrates and one non-substrate 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), non-substrate 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). ●, basal-to-apical transport across the cynomolgus monkey P-gp-transfected LLC-PK1 cell monolayer; ■, apical-to-basal transport across the cynomolgus monkey P-gp-transfected LLC-PK1 cell monolayer; ○, basal-to-apical transport across the parental LLC-PK1 cell monolayer; □, apical-to-basal transport across the parental LLC-PK1 cell monolayer.

Figure 2: Comparison of the observed and reconstructed $K_p$brain (A) and $K_{p,uu,brain}$ (B) for the six compounds. These data were taken from Tables 1 and 3. The solid line passing through the origin represents the line of identity, and the broken lines represent three-fold differences. Each point represents the mean ± S.E.M.. 1, indinavir; 2, quinidine; 3, loperamide; 4, paclitaxel; 5, diazepam; and 6, verapamil.

Figure 3: Comparison of the observed and reconstructed species differences in the $K_p$brain (A) and $K_{p,uu,brain}$ (B) of the six compounds between cynomolgus monkeys and mice. The cynomolgus monkey data were taken from Tables 1, 3, and 4 and then divided by the mouse data cited in Uchida et al. (2011a). The solid line passing through the origin represents the line of identity, and the broken lines represent three-fold differences. 1, indinavir; 2, quinidine; 3, loperamide; 4, paclitaxel; 5, diazepam; and 6, verapamil.
Table 1: Time profiles of the plasma concentrations and $K_{p\,\text{brain}}$ values of the six compounds in male cynomolgus monkeys after intravenous constant infusion

The male cynomolgus monkeys (fasted overnight) were fixed to monkey chairs without anesthesia during compound administration. Indinavir, quinidine, diazepam, and verapamil were intravenously infused for 3 h at dose rates of 0.61, 0.21, 0.070, and 0.40 mg/h/kg, 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 h at dose rates of 0.20 and 0.94 mg/h/kg, respectively, without intravenous bolus injections. The right cerebrums were used to determine the brain-to-plasma concentration ratios ($K_{p\,\text{brain}}$) at 3 h. One cynomolgus monkey was used for each compound.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Plasma Concentration (μM)</th>
<th>$K_{p,\text{brain}}$ (mL/g brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>2</td>
</tr>
<tr>
<td>Indinavir</td>
<td>BLQ</td>
<td>0.525</td>
</tr>
<tr>
<td>Quinidine</td>
<td>BLQ</td>
<td>0.482</td>
</tr>
<tr>
<td>Loperamide</td>
<td>BLQ</td>
<td>0.0699</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>BLQ</td>
<td>0.0779</td>
</tr>
<tr>
<td>Diazepam</td>
<td>BLQ</td>
<td>0.0859</td>
</tr>
<tr>
<td>Verapamil</td>
<td>BLQ</td>
<td>0.601</td>
</tr>
</tbody>
</table>

Pre, just prior to the administration of the compounds;

BLQ, below the limit of quantification
Table 2: Reconstruction of P-gp activities at the cynomolgus monkey BBB based on the in-vitro transport activities and protein expression levels

Based on 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 the Materials and Methods section. 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 compound, the cynomolgus monkeys were sacrificed, the cerebrums were collected, and the left cerebrums were used to isolate brain microvessels using the nylon mesh method. P-gp protein expression levels in the brain microvessels of each cynomolgus monkey were determined using LC-MS/MS-based quantification (triplicate experiments for each cynomolgus monkey). Reconstructed $K_p$ brain ratios were calculated from the in-vitro P-gp efflux 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>
<thead>
<tr>
<th>Compound</th>
<th>LLC-PK1 Transport rate (fmol/min/well)</th>
<th>Flux ratio</th>
<th>Cynomolgus monkey P-gp-transfected LLC-PK1 Transport rate (fmol/min/well)</th>
<th>Flux ratio</th>
<th>In-vitro P-gp efflux ratio</th>
<th>Protein expression levels of cynomolgus monkey P-gp (fmol/μg protein)</th>
<th>Cynomolgus monkey P-gp-transfected LLC-PK1 Brain microvessels isolated from cynomolgus monkey</th>
<th>Reconstructed $K_p$ brain ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indinavir</td>
<td>88.2 ± 0.5</td>
<td>298 ± 19</td>
<td>3.37 ± 0.15</td>
<td>33.8 ± 1.1</td>
<td>320 ± 20</td>
<td>9.47 ± 0.47</td>
<td>2.81 ± 0.13</td>
<td>2.31 ± 0.15</td>
</tr>
<tr>
<td>Quinidine</td>
<td>338 ± 34</td>
<td>744 ± 84</td>
<td>2.21 ± 0.24</td>
<td>72.8 ± 8.4</td>
<td>786 ± 79</td>
<td>10.8 ± 1.2</td>
<td>4.90 ± 0.53</td>
<td>2.31 ± 0.15</td>
</tr>
<tr>
<td>Loperamide</td>
<td>79.8 ± 4.1</td>
<td>60.3 ± 2.6</td>
<td>0.755 ± 0.036</td>
<td>30.0 ± 2.8</td>
<td>181 ± 13</td>
<td>6.04 ± 0.49</td>
<td>8.00 ± 0.53</td>
<td>2.31 ± 0.15</td>
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<tr>
<td>Paclitaxel</td>
<td>98.7 ± 6.3</td>
<td>425 ± 27</td>
<td>4.31 ± 0.28</td>
<td>33.8 ± 4.2</td>
<td>577 ± 32</td>
<td>17.1 ± 1.6</td>
<td>3.96 ± 0.32</td>
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<td>Diazepam</td>
<td>723 ± 25</td>
<td>938 ± 41</td>
<td>1.30 ± 0.05</td>
<td>937 ± 64</td>
<td>1197 ± 61</td>
<td>1.28 ± 0.08</td>
<td>0.984 ± 0.050</td>
<td>2.31 ± 0.15</td>
</tr>
<tr>
<td>Verapamil</td>
<td>163 ± 6</td>
<td>246 ± 8</td>
<td>1.51 ± 0.05</td>
<td>92.6 ± 3.7</td>
<td>394 ± 21</td>
<td>4.26 ± 0.20</td>
<td>2.83 ± 0.12</td>
<td>2.31 ± 0.15</td>
</tr>
</tbody>
</table>

*Previously reported in Ito et al. (2011) and cited from that study.
Table 3: Reconstruction of the $K_p$ brain of the six compounds in cynomolgus monkeys based on the in-vitro data

Cynomolgus monkey plasma was spiked with 500 nM concentrations of the compounds and dialyzed against PBS (pH 7.4) at 37°C for 6 h ($n = 3$). One-quarter of the diluted brain homogenates of the cynomolgus monkeys was spiked with 260 nM indinavir, 559 nM quinidine, 870 nM loperamide, 3,110 nM paclitaxel, 333 nM diazepam, and 117 nM verapamil and dialyzed against ECF buffer (pH 7.4) at 37°C for 6 h to obtain the homogenate $f_{u,brain}$ ($n = 3$). The $f_{u,brain}$ (homogenate + pH partition model) was calculated from the homogenate $f_{u,brain}$ in combination with the reported pH partition model (Friden et al., 2011) using the reported pKa values of the compounds (Carvalho-Silva et al., 2004; Friden et al., 2011). The reconstructed $K_p$ brain values were calculated from the reconstructed $K_p$ brain ratios (see Table 2) and the $f_{u,plasma}$ and $f_{u,brain}$ (homogenate + pH partition model) values using eq. 3. 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>$f_{u,plasma}$</th>
<th>$f_{u,brain}$</th>
<th>Reconstructed $K_p$ brain (mL/g brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homogenate</td>
<td>Homogenate + pH partition model</td>
</tr>
<tr>
<td>Indinavir</td>
<td>0.643 ± 0.044</td>
<td>0.297 ± 0.021</td>
<td>0.261 ± 0.018</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.0591 ± 0.0015</td>
<td>0.0621 ± 0.0049</td>
<td>0.0219 ± 0.0017</td>
</tr>
<tr>
<td>Loperamide</td>
<td>0.0776 ± 0.0052</td>
<td>0.0103 ± 0.0012</td>
<td>0.00360 ± 0.00042</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.125 ± 0.006</td>
<td>0.0151 ± 0.0014</td>
<td>0.0151 ± 0.0014</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.0523 ± 0.0025</td>
<td>0.0356 ± 0.0014</td>
<td>0.0356 ± 0.0014</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.211 ± 0.013</td>
<td>0.0416 ± 0.0031</td>
<td>0.0145 ± 0.0011</td>
</tr>
</tbody>
</table>
Table 4: Reconstructed and estimated in-vivo $K_{pu,brain}$ values of the six compounds in cynomolgus monkeys

The reconstructed $K_{pu,brain}$ values were calculated as the reciprocals of the reconstructed $K_p$ brain ratios (see Table 2) using eq. 5. The in-vivo $K_{pu,brain}$ values were estimated from the $f_u,plasma$ and $f_u,brain$ (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 $K_{pu,brain}$ Value</th>
<th>Estimated in-vivo $K_{pu,brain}$ 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>
Table 5: Impact of four parameters on species differences in the $K_p$ brain and $K_{p,uu,brain}$ values between cynomolgus monkeys and mice

The contributions of individual parameters (P-gp protein expression levels at the BBB, the intrinsic transport activity per P-gp molecule, and the unbound fractions in the plasma and brain) to the species differences in the $K_p$ brain and $K_{p,uu,brain}$ values between cynomolgus monkeys and mice are presented. $PL_{p-gp,vivo}$ represents the protein expression levels of P-gp in isolated brain microvessels. $TA_{int,p-gp}$ represents the intrinsic transport activity per P-gp molecule and is calculated as $[(In-vitro P-gp efflux ratio) - 1]/[P-gp protein expression levels in P-gp-transfected LLC-PK1 cells]$. The values of $PL_{p-gp,vivo}$, $TA_{int,p-gp}$, $f_{u,plasma}$, and $f_{u,brain}$ in cynomolgus monkeys were divided by the corresponding values in mice to obtain the “Monkey/Mouse” values for each compound. The cynomolgus monkey data were taken from Tables 2 and 3, and the mouse data were cited from Uchida et al. (2011a). The values of the “Impact on $K_p,monkey/K_p,mouse$” and the “Impact on $K_{p,uu,monkey}/K_{p,uu,mouse}$” describe the contributions of individual parameters to the species differences in the $K_p$ brain and $K_{p,uu,brain}$ values between cynomolgus monkeys and mice and were calculated using eq. 10 and 11, respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PL_{p-gp,vivo}</th>
<th>TA_{int,p-gp}</th>
<th>f_{u,plasma}</th>
<th>f_{u,brain}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indinavir</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/Mouse</td>
<td>0.414</td>
<td>1.39</td>
<td>11.1</td>
<td>3.19</td>
</tr>
<tr>
<td>Impact on $K_p,monkey/K_p,mouse$</td>
<td>2.16</td>
<td>0.768</td>
<td>11.1</td>
<td>0.313</td>
</tr>
<tr>
<td>Impact on $K_{p,uu,monkey}/K_{p,uu,mouse}$</td>
<td>2.16</td>
<td>0.768</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Quinidine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/Mouse</td>
<td>0.406</td>
<td>0.802</td>
<td>0.369</td>
<td>0.535</td>
</tr>
<tr>
<td>Impact on $K_p,monkey/K_p,mouse$</td>
<td>2.33</td>
<td>1.22</td>
<td>0.369</td>
<td>1.87</td>
</tr>
<tr>
<td>Impact on $K_{p,uu,monkey}/K_{p,uu,mouse}$</td>
<td>2.33</td>
<td>1.22</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Loperamide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/Mouse</td>
<td>0.358</td>
<td>1.92</td>
<td>3.37</td>
<td>0.752</td>
</tr>
<tr>
<td>Impact on $K_p,monkey/K_p,mouse$</td>
<td>2.68</td>
<td>0.550</td>
<td>3.37</td>
<td>1.33</td>
</tr>
<tr>
<td>Impact on $K_{p,uu,monkey}/K_{p,uu,mouse}$</td>
<td>2.68</td>
<td>0.550</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Paclitaxel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/Mouse</td>
<td>0.370</td>
<td>1.72</td>
<td>5.95</td>
<td>3.09</td>
</tr>
<tr>
<td>Impact on $K_p,monkey/K_p,mouse$</td>
<td>2.48</td>
<td>0.635</td>
<td>5.95</td>
<td>0.323</td>
</tr>
<tr>
<td>Impact on $K_{p,uu,monkey}/K_{p,uu,mouse}$</td>
<td>2.48</td>
<td>0.635</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Diazepam</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/Mouse</td>
<td>0.501</td>
<td>-</td>
<td>0.534</td>
<td>0.615</td>
</tr>
<tr>
<td>Impact on $K_p,monkey/K_p,mouse$</td>
<td>1</td>
<td>1</td>
<td>0.534</td>
<td>1.63</td>
</tr>
<tr>
<td>Impact on $K_{p,uu,monkey}/K_{p,uu,mouse}$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Verapamil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey/Mouse</td>
<td>0.369</td>
<td>0.979</td>
<td>1.92</td>
<td>0.564</td>
</tr>
<tr>
<td>Impact on $K_{p,\text{monkey}}/K_{p,\text{mouse}}$</td>
<td>2.38</td>
<td>1.02</td>
<td>1.92</td>
<td>1.77</td>
</tr>
<tr>
<td>Impact on $K_{p,u,\text{monkey}}/K_{p,u,\text{mouse}}$</td>
<td>2.38</td>
<td>1.02</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

(A) $K_p^{\text{brain}}$

![Graph showing the relationship between reconstructed $K_p^{\text{brain}}$ (mL/g brain) and observed $K_p^{\text{brain}}$ (mL/g brain).]

(B) $K_p^{\text{uu,brain}}$

![Graph showing the relationship between reconstructed $K_p^{\text{uu,brain}}$ (mL/g brain) and estimated in-vivo $K_p^{\text{uu,brain}}$.]

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*Downloaded from jpet.aspetjournals.org on July 9, 2017.*
Figure 3

A) $\frac{K_{p,\text{monkey}}}{K_{p,\text{mouse}}}$

B) $\frac{K_{p,\text{uu,monkey}}}{K_{p,\text{uu,mouse}}}$