Prediction of Drug Permeability Using In Vitro Blood–Brain Barrier Models with Human Induced Pluripotent Stem Cell-Derived Brain Microvascular Endothelial Cells

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Abstract
The strong barrier function of the blood–brain barrier (BBB) protects the central nervous system (CNS) from xenobiotic substances, while the expression of selective transporters controls the transportation of nutrients between the blood and brain. As a result, the delivery of drugs to the CNS and prediction of the ability of specific drugs to penetrate the BBB can be difficult. Although in vivo pharmacokinetic analysis using rodents is a commonly used method for predicting human BBB permeability, novel in vitro BBB models, such as Transwell models, have been developed recently. Induced pluripotent stem cells (iPSCs) have the potential to differentiate into various types of cells, and protocols for the differentiation of iPSCs to generate brain microvascular endothelial cells (BMECs) have been reported. The use of iPSCs makes it easy to scale-up iPSC-derived BMECs (iBMECs) and enables production of BBB disease models by using iPSCs from multiple donors with disease, which are advantageous properties compared with models that utilize primary BMECs (pBMECs). There has been little research on the value of iBMECs for predicting BBB permeability. This study focused on the similarity of iBMECs to pBMECs and investigated the ability of iPSC-BBB models (monoculture and coculture) to predict in vivo human BBB permeability using iBMECs. iBMECs express BMEC markers (e.g., VE-cadherin and claudin-5) and influx/efflux transporters (e.g., Glut-1, SLC7A5, CD220, P-gp, ABCG2, and MRP-1) and exhibit high barrier function (transendothelial electrical resistance, >1000 Ω·cm²) as well as similar transporter expression profiles to pBMECs. We determined that the efflux activity using P-glycoprotein (P-gp) transporter is not sufficient in iBMECs, while in drug permeability tests, iPSC-derived BBB models showed a higher correlation with in vivo human BBB permeability compared with a rat BBB model and the Caco-2 model. In a comparison between monoculture and coculture models, the coculture BBB model showed higher efflux activity for compounds with low CNS permeability (e.g., verapamil and thioridazine). In conclusion, iPSC-BBB models make it possible to predict BBB permeability, and employing coculturing can improve iPSC-BBB function.

Keywords: drug discovery; neurotherapeutic drugs; neuron; astrocyte

Introduction
Clinical trials of central nervous system (CNS) drugs have shown a very low overall success rate (6.2% vs. 13.3% for non-CNS drugs) and the time required for approval by the U.S. Food and Drug Administration (FDA) is longer (19.3 vs. 14.7 months for non-CNS drugs).1 The major reasons for failures in CNS drug development are (1) unknown drug distribution in the CNS and (2) a gap between pre-clinical and clinical data due to interspecies variation.2 One of the challenges...
in resolving these issues is the development of in vitro assays, as an alternative to animal models, which can accurately estimate pharmacokinetics in the CNS. 

In silico simulation models have been established to predict in vivo blood–brain barrier (BBB) permeability. The accuracy of in silico prediction of BBB permeability to small molecules (<1000 Da) has improved; however, it remains difficult to predict BBB permeability to noncovalent, inorganic, higher molecular weight, and mixtures of compounds using this model.

The BBB is a key structure in the CNS for nutrients and drugs to penetrate from the blood vessels to the brain cortex, and the tight junctions (TJs) formed by brain microvascular endothelial cells (BMECs) have a high barrier function. 

The high barrier function of BMECs hinders the ability of neurotherapeutic drugs to exert therapeutic effects in the CNS. To investigate CNS pharmacokinetics, alternate assays, as an alternative to animal models, which can accurately estimate pharmacokinetics in the CNS. 

Therefore, in this study, we therefore investigated whether an in vitro BBB model using iBMECs can predict human in vivo drug permeability in the CNS.

### Materials and Methods

#### Differentiation of BMECs from iPSCs

iBMECs were differentiated from human iPSCs using differentiation protocols reported by Shusta and others, with minor modifications. iPSCs (01279 line provided by FUJIFILM Cellular Dynamics, Inc. [FCDI], W1) were cultured on vitronectin-coated plates (1:100; Thermo Fisher Scientific, Inc., MA) with Essential 8 Flex Medium (E8; Gibco, MA) in an incubator at 37°C with 5% O2. To induce BMEC differentiation, 14,000 cells/cm² of singularized iPSCs were seeded on a vitronectin-coated T150 flask in E8 with Y-27632 (10 μM; FUJIFILM Wako Pure Chemical Co. [Wako], Japan) on day 0 (D0). E8 was replaced with unconditioned medium (UM) containing 20% KnockOut Serum Replacement (Gibco), 1×MEM nonessential amino acids (Gibco), 1 mM L-glutamine (Sigma-Aldrich, MO), and 0.1 mM β-mercaptoethanol (Wako) in DMEM/F12 (Gibco) on D1, and the UM was refreshed every day from D1–D4. Cells were subsequently cultured in human endothelial cell medium (ECM; Gibco) containing 1% platelet-poor plasma (PPP) serum (bovine derived; Thermo Fisher Scientific, Inc.; human-derived; Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, MN) and 10 μM retinoic acid (Sigma-Aldrich) from D5–D8, and the ECM was refreshed every other day. Cells were then harvested and seeded on Transwells (φ40 μm; Millipore, MA; or Transwell Permeable Supports for immunocytochemistry [ICC]; Corning, NY) coated with 0.1 mg/mL fibronectin (Wako) and 0.1 mg/mL CellMatrix Type IV (Nitta Gelatin, Japan) at 1.0 × 10⁶ cells/cm² in ECM, and the medium was replaced with ECM minus bFGF on D9. Cells were maintained in 5% O2 from D0–D8, and transferred to normoxic conditions from D8 after subculturing on Transwells. On D10 and D11, the induced BMECs were subjected to TEER measurement, ICC staining, mRNA extraction, and drug permeability assay (Fig. 1A).
Coculture with astrocytes and neurons

For coculturing, astrocytes (0.12 × 10^6 cells/cm², iCell Astrocyte; FCDI) and neurons (0.04 × 10^6 cells/cm², iCell GABA Neuron; FCDI) were cocultured on 24-well plates coated with 0.1 mg/mL poly-l-lysine (TREVIGEN, MD) and 3.3 lg/mL laminin-521 (BioLamina, Sweden) in 1:1 astrocyte/neuron medium purchased from FCDI on D4. On D8, 5.0 × 10^3 cells/cm² of primary human brain vascular pericytes (ScienCell, CA) were seeded on the basolateral side of Transwells by flipping the plate upside-down in a pericyte medium on D7. BMECs were seeded on the upper side of the Transwells with pericytes and cultured with astrocytes/neurons on the basolateral side with either ECM or neuron medium from D8–D11. TEER was measured on D10 and D11, ICC was performed on D10, and drug permeability tests and mRNA were performed on D11. (B) Structures of hiBBB models. ECM is used in apical wells in all monoculture and coculture models. For Mono-ECM and Co-ECM, ECM was used in the basolateral wells, and for Mono-N and Co-N, neuron medium was used in the basolateral wells. bFGF, basic fibroblast growth factor; BMEC, brain microvascular endothelial cell; ECM, endothelial cell medium; hiBBB, human iPSC-derived blood–brain barrier; ICC, immunocytochemistry; iPSC, induced pluripotent stem cell; RA, retinoic acid; TEER, transendothelial electrical resistance.

FIG. 1. Schema of experimental protocols. (A) iPSCs were seeded on D0 and cultured in unconditional medium from D1–D5 and in ECM (+RA, +bFGF) from D5–D8 to differentiate BMECs. On D8, BMECs were subcultured on Transwells in ECM (+RA, +bFGF) on the apical side and the medium was changed to ECM (+RA) on D9. For monoculture, either ECM (+RA) or neuron medium was used in basolateral wells. For coculture, astrocytes and neurons were cultured in astrocyte/neuron medium (1:1) from D4–D8 and pericytes were seeded on the back side of the Transwell in pericyte medium on D7. BMECs were seeded on the upper side of the Transwells with pericytes and cultured with astrocytes/neurons on the basolateral side with either ECM or neuron medium from D8–D11. TEER was measured on D10 and D11, ICC was performed on D10, and drug permeability tests and mRNA were performed on D11. (B) Structures of hiBBB models. ECM is used in apical wells in all monoculture and coculture models. For Mono-ECM and Co-ECM, ECM was used in the basolateral wells, and for Mono-N and Co-N, neuron medium was used in the basolateral wells. bFGF, basic fibroblast growth factor; BMEC, brain microvascular endothelial cell; ECM, endothelial cell medium; hiBBB, human iPSC-derived blood–brain barrier; ICC, immunocytochemistry; iPSC, induced pluripotent stem cell; RA, retinoic acid; TEER, transendothelial electrical resistance.

TEER measurement

TEER was measured on D10 and D11 using an EVOM2 voltohmeter with ENDOHM-6 chambers (World Precision Instruments, FL). The TEER was recorded at the peak value. Membrane resistance was calculated as TEER (Ω × cm²) = measured resistance value (Ω) × surface area (cm²).

ICC staining

ICC was performed using an Image-iT Kit (Invitrogen, MA) on D10. Briefly, iBMECs on permeable Transwells were washed with Dulbecco’s phosphate-buffered saline
(DPBS) and fixed in 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.5% Triton X-100 in DPBS for 10 min and blocking with Blocking Solution for 60 min. For staining, cells were incubated with primary antibodies (Table 1) overnight at 4°C, and subsequently stained with donkey anti-mouse IgG Alexa Fluor 488 or donkey anti-rabbit IgG Alexa Fluor 594 (1:1000; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:10,000; Biotium, CA) for 15 min. Protein expression was visualized by fluorescence microscopy (BZ-X710; KEYENCE, Japan).

Caco-2 and ratBBB kits
To compare drug permeability with non-BMEC and non-human cells, we performed drug permeability tests using Caco-2 human epithelial colorectal adenocarcinoma cells and rat pBMECs. A Caco-2 kit (KAC Co. Ltd., Japan) was used according to the manufacturer’s instructions. Briefly, the medium was refreshed and the kits were incubated overnight and used for permeability tests the next day.

RatBBB kits (RBE-12; PharmaCo-Cell Company Ltd., Japan) were maintained according to the manufacturer’s instructions. Briefly, the kits were thawed by adding thawing solution and subsequently cultured with Culture medium 1 (500 μL in apical well and 1500 μL in basolateral well). After overnight incubation, the medium was replaced with Culture medium 2 and the cells were maintained for 4 days before use in drug permeability tests. Both drug permeability tests using Caco-2 and ratBBB kits were performed following the protocol described for drug permeability tests in the Material and Methods section.

Drug transporter array
All reagents and array plates were purchased from Qiagen-SABioscience. Cells were extracted with lysis buffer on D11, homogenized with a QIAshredder, and mRNA was purified using an RNeasyPlus Micro Kit with DNaseI. Transcription of CDNA from mRNA was performed using an RT² First Strand Kit, and human drug transporter array (RT² Profiler PCR Array Human Drug Transporters, 330231 PAHS-070Z) was performed with RT² SYBR Green qPCR Mastermixes. All procedures were performed according to the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) was performed on a ViiA 7 Real-Time PCR System, and data analysis was performed using R statistical software (version 3.5.2). Heat map data were drawn based on ΔCt values normalized to the average of eight housekeeping genes (i.e., Tap1, Tap2, Vdac1, Vdac2, B2m, GAPDH, Hprt1, and RPLPO) and Ward’s clustering method was used for the analysis.

Drug permeability tests
Cells were incubated with Hank’s Balanced Salt Solution (HBSS) (Wako) for 2 h on D11 and the TEER was measured before carrying out permeability tests. hiBBB models with barrier function less than 500 Ω × cm² were excluded from drug permeability tests. For Caco-2 and ratBBB kits, all Transwells were used for permeability tests. Drugs at a concentration of 10 μM (Tables 2 and 3) diluted in HBSS were added to apical wells for A-to-B samples or basolateral wells for B-to-A samples; the cells were then incubated at 37°C with agitation and 50-μL samples from the basolateral wells (A-to-B samples) and apical wells (B-to-A samples) were collected at 60 and 80 min. Drug concentrations were measured by LC/MS analysis (LC: Prominence, MS: LCMS-2010EV; Shimazu Co., Japan), and LC/MSMS analysis (LC: Acquity; Waters Co., MA; MS: TSQ QUANTIVA; Thermo Fisher Scientific, Inc.) was used for digoxin measurement. For the

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**Table 1. Antibodies**

| Antibodies | Host | Dilution | Code no. | Supplier |
|------------|------|----------|----------|----------|
| VE-cadherin | Rabbit | 1:50 | ab33168 | Abcam, UK |
| vWF | Rabbit | 1:50 | ab6994 | Abcam |
| Ulex | Rabbit | 1:50 | FL-1061-2 | Vector Laboratories, Inc., CA |
| Glut-1 | Mouse | 1:50 | MS-10637-P0 | Thermo Fisher Scientific, Inc. |
| CD220 | Mouse | 1:50 | MA5-13778 | Invitrogen |
| MRP-1 | Mouse | 1:25 | MAB4155 | Millipore |
| P-gp | Rabbit | 1:50 | ab170904 | Abcam |
| SLC7A5 | Rabbit | 1:100 | HPA052673 | Atlas Antibodies, Sweden |
| ABCG2 | Mouse | 1:50 | MAB4100 | Millipore |
| Occludin | Mouse | 1:50 | 33-1500 | Invitrogen |
| Claudin-5 | Mouse | 1:50 | 35-2500 | Invitrogen |
| ZO-1 | Rabbit | 1:50 | 61-7300 | Invitrogen |

**Table 2. Drug Permeability test 1: P-Glycoprotein Substrates**

| Drugs | MW | Solvent | Code no. | Suppliers |
|-------|----|---------|----------|-----------|
| Digoxin | 780.9 | DMSO | B21902 | Alfa Aesar, UK |
| Colchicine | 399.4 | DMSO | 039-03851 | Wako |
| Quinidine | 324.4 | DMSO | 176-00111 | Wako |
| Vinblastine | 909.1 | DMSO | 11762 | Cayman Chemical, MI |
| Gilbenclamide | 494.0 | DMSO | 078-03881 | Wako |
| Caffeine (simple diffusion) | 194.2 | DMSO | C0750 | Sigma-Aldrich |

DMSO, dimethyl sulfoxide; MW, molecular weight.
data analyses, Papp (apparent permeability coefficients; cm/sec), and efflux ratios were calculated using the following formulas:

\[
Papp (cm/sec) = \frac{dQ}{dT} \times A \times C_0
\]

\[
\text{Efflux ratio} = \frac{Papp(B - to - A)}{Papp(A - to - B)}
\]

where \(dQ/dT\) is the amount of drug transported per unit time; \(A\) is the membrane surface area; and \(C_0\) is the donor concentration at time 0.

Statistics
Comparisons of more than three groups were performed using two-way analysis of variance (ANOVA) followed by Tukey’s–Kramer test. For TEER measurement, two-way repeated measures ANOVA followed by Bonferroni–Dunn test was used. Statistical significance was expressed as *\(p < 0.05\) and **\(p < 0.01\). Statcel3 software (OMS Publishing, Inc., Japan) was used for all statistical analyses. All values are presented as mean ± standard deviation, unless otherwise indicated.

Results
Barrier function
We initially compared the effects of PPP in the Mono-ECM model between bovine (bPPP) and human derived (hPPP). hPPP remarkably improved the differentiation efficiency compared with bPPP (100% in hPPP vs. 66.7% in bPPP), and moreover, significantly increased the barrier function compared with the use of bPPP on D9 (hPPP, 1223 ± 406, \(n = 24\), vs. bPPP, 919 ± 306, \(n = 48\), \(\Omega \times cm^2\), \(p < 0.05\)); we therefore used hPPP in subsequent ECM model experiments. All hiBBB models showed remarkably high barrier function >1000 \(\Omega \times cm^2\). Neuron medium in basolateral wells significantly increased the TEER compared with ECM in both monoculture and coculture models on D10 (Mono-N, 1920 ± 774, \(n = 20\); Co-N, 1908 ± 582, \(n = 23\) vs. Mono-ECM, 1423 ± 592, \(n = 24\); Co-ECM, 1454 ± 263, \(n = 7\); \(\Omega \times cm^2\)). Caco-2 and ratBBB showed significantly lower TEER values compared with pBMECs (Caco-2, 239 ± 97, \(n = 20\); ratBBB, 425 ± 67, \(n = 24\), on D10; \(\Omega \times cm^2\)). Taken together, these results suggest the neuron medium greatly improved the barrier function of the hiBBB model (Fig. 2A).

BMEC markers in iBMECs
iBMECs, differentiated according to the protocol employed, expressed EC markers (i.e., VE-cadherin, vWF, and Ulex), Tjs proteins (i.e., claudin-5, occluding and ZO-1) (Fig. 2B). All groups showed similar expression of influx transporters (i.e., Glut-1, SLC7A5, and CD220) and efflux transporters (i.e., P-pg, ABCG2 and MRP-1)\(^{10}\) (Fig. 2C). These results demonstrated that both monoculture with neuron medium in basolateral wells and coculture with pericytes, astrocytes, and neurons did not affect the expression of BMEC markers.

Drug transporter expression
Transporter expression profiles of iBMECs (\(n = 3\)) were compared with pBMECs (\(n = 1\)), Caco-2 cells (\(n = 1\)), iPSCs (\(n = 2\)), and human umbilical cord blood cells (HUVECs, \(n = 1\)). In contrast to HUVECs, iPSCs, and Caco-2 cells, the hiBBB models showed very similar expression profiles to that of pBMECs. In comparison to hPPP (Mono-hECM) and bPPP (Mono-bECM), pBMEC showed a closer expression profile to Mono-hECM than to Mono-bECM. There was almost no difference in transporter expression among the hiBBB models. With respect to typical efflux transporters, Caco-2 cells clearly showed higher expression of P-gp/ABCB1 versus other cell types, while ABCC1/BCRP expression was almost the same among pBMECs, hiBBB models, and Caco-2 cells, and ABCG2/MRP-1 expression was the same between pBMEC and hiBBB models, but lower in Caco-2 cells (Fig. 3).

### Table 3. Drug Permeability Test 2: Comparison with Human In Vivo Permeability

| Drugs       | MW  | Solvent | Code no. | Suppliers                          | CSF/plasma in human<sup>27</sup> |
|-------------|-----|---------|----------|-----------------------------------|----------------------------------|
| Bupropion   | 276.2 | H<sub>2</sub>O | 028-17311 | Wako                              | 0.43                             |
| Gabapentin  | 171.2 | H<sub>2</sub>O | 076-05641 | Wako                              | 0.113                            |
| Lamotrigine | 255.0 | DMSO    | L0349    | LKT Laboratories, Inc., MI        | 0.43                             |
| Tacrine     | 234.7 | DMSO    | 70240    | Cayman Chemical                   | 0.39                             |
| Thioridazine| 407.0 | DMSO    | 79025    | Sigma-Aldrich                     | 0.01                             |
| Topiramate  | 339.4 | DMSO    | TS40250  | Toronto Research Chemicals, Canada | 0.84                             |
| Verapamil   | 454.6 | H<sub>2</sub>O | V4629    | Sigma-Aldrich                     | 0.068                            |

CSF, cerebrospinal fluid.

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Drug permeability tests
We investigated drug permeability of Co-N, which most closely mimics the in vivo properties of the BBB and has frequently been used as an in vitro BBB model, comparing Mono-bECM, reported by Lippmann et al. as the original iBMECs, comparing Caco-2 and ratBBB kits. We first tested the drugs listed under permeability test 1 of Table 2, which have been reported to be P-glycoprotein (P-gp) substrates. Caffeine was used as a positive control for simple diffusion across the BBB and it showed high permeability in all in vitro BBB models. Mono-ECM showed a remarkably high
efflux ratio in vinblastine and digoxin, and showed <2.0 efflux ratio in quinidine, colchicine, and glibenclamide.

Co-N showed >2.0 high efflux ratio in glibenclamide, vinblastine, and digoxin, and showed <2.0 efflux ratio in quinidine and colchicine. Caco-2 cells showed high efflux activity for all P-gp substrates (Fig. 4A). We subsequently investigated the correlation between in vivo human drug permeability (Table 3, permeability test 2) and drug permeability of iBMECs (Mono-ECM and Co-N) compared with ratBBB and Caco-2 kits. hiBBB models showed a greater correlation with in vivo data compared with Caco-2 kit, with an efflux ratio greater than two for gabapentin, verapamil, and thioridazine, which are reported to have low CNS permeability (Mono-ECM, $R^2 = 0.49$; Co-N, $R^2 = 0.60$; Caco-2 kit, $R^2 = 0.41$). RatBBB kit showed higher correlation than hiBBB (ratBBB kit, $R^2 = 0.73$); however, high concentration of verapamil and gabapentin penetrated to basolateral well across ratBMECs, which indicates that the ratBBB kit is inappropriate as a drug permeability prediction tool in substrates for efflux transporter. Taken together, these results suggest that P-gp transporter expression is insufficient in hiBBB models; however, hiBBB models have a better ability to predict drug permeability compared with other prediction models.

**Discussion**

Although some reports have indicated benefits of using iBMECs for in vitro BBB modeling, the degree of similarity between iBMECs and pBMECs is not well known. We therefore focused on the ability of hiBBB models to predict in vivo drug permeability, and the transporter expression profiles of iBMECs compared with pBMECs. We first confirmed that iBMECs express BMEC markers (i.e., Tjs, EC marker, and influx/efflux transporters). In addition, this study demonstrated that neuron medium improves barrier function without coculturing, and that hPPP improves the reproducibility of iBMEC differentiation. Given that PPP contains

**FIG. 3.** Drug transporter array heat map. Mono-hECM showed the highest similarity to the transporter expression profiles of pBMECs. All hiBBB models showed similar expression profiles to pBMEC. Caco-2 cells, iPSCs, and HUVECs had expression profiles that differed between the pBEMC and hiBBB models. Data are shown as ΔCt. HUVEC, human umbilical cord blood cell; pBMEC, primary BMEC.
angiogenic factors such as vascular endothelial growth factor, bFGF, platelet-derived growth factor, angiopoietin, and epidermal growth factor, the robustness of BMEC differentiation might be affected by PPP's angiogenic potential, as suggested by the difference between hPPP and bPPP. Indeed, in drug transporter array, pBMEC had a closer expression profile to Mono-hECM than to Mono-bECM, which indicates that the formulation of PPP is crucial for controlling BMEC function. Using the method of Lippoman et al., we succeeded in shortening the differentiation period and improving the robustness of BMEC differentiation.

Transporter expression profiles in iBMECs were similar to pBMECs, while the expression of some transporters was remarkably higher or lower in iBMECs. Higher expression in iBMEC was mostly observed for the SLC2 family of glucose transporters (i.e., SLC2A1 and SLC2A3) and the SLC22 family, which transports organic anions/cations (i.e., SLC22A3 and SLC22A8); however, there was no consistent pattern. P-gp on BMECs is a gatekeeper protein for xenobiotics and serves as an essential transporter to protect the CNS; however, P-gp expression was low in iBMECs, even though ICC clearly demonstrated P-gp expression. In iBMECs, the insufficient expression of P-gp is one of the features that needs to be improved upon to accurately predict the permeability of P-gp substrates. Indeed, hiBBB models (e.g., Mono-ECM and Co-N) fail to exert efflux activity with some P-gp substrates such as quinidine, colchicine, and glibenclamide. In contrast, these models showed sufficiently high efflux activity in digoxin, verapamil, and vinblastine, which are known to be P-gp substrates. Since the transporter expression profiles of in vivo BMECs remain unclear, it is important to more thoroughly understand the transporter expression of in vivo BMECs, and manipulating the transporter expression of iBMECs to be comparable to that of in vivo BMECs is crucial for developing in vivo-like hiBBB models.

To assess the ability of in vitro BBB models to predict drug permeability, we selected drugs for which in vivo pharmacodynamics in the human CNS have been previously established. Both ratBBB and Caco-2 kits showed poor predictive ability, while hiBBB models...
(both monoculture and coculture) correlated with human in vivo permeability. Drugs with high BBB permeability, such as topiramate, bupropion, lamotrigine, and tacrine, showed the same permeation ratios among the groups tested. Although transporters for topiramate, bupropion, lamotrigine, and tacrine have been identified as P-gp, dopamine transporter, organic cation transporter I (OCT1), and choline transporter, respectively, not all transporters for these drugs have been determined. In this study, their high distribution in the CSF, as shown in in vivo studies, was confirmed by the high permeability observed in the hiBBB models. Gabapentin is a substrate for SLC7A5 influx transporter, which is known to localize at the BBB; however, based on in vivo studies, it shows poor distribution in the CNS. The drug transporter array showed, in iBMECs, the extremely high SLC7A5 expression, while the permeability of gabapentin was poor in the hiBBB model employing iBMECs. Thus, transporter expression and in vivo permeability do not necessarily correspond. Verapamil is widely known as an efflux compound by P-gp, and hiBBB models exhibited efflux activity for verapamil, while both ratBBB kit and Caco-2 kits showed low efflux activity for verapamil. Taken together, these results clearly indicate that drug permeability in hiBBB models connotes mechanisms implicating efflux transport of gabapentin, verapamil, and thioridazine.

Conclusion
The hiBBB model is more reliable for predicting drug permeability compared with non-human and non-BMEC BBB models. Further elucidation of transport mechanisms by the BBB is essential for improving predictive accuracy.

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Authors’ Contributions
M.O.: conception and design, collection and/or assembly of data, data analysis, and interpretation. Article writing: S.K.: collection and/or assembly of data, data analysis, and interpretation; H.F.: data analysis; S.M.: article writing and interpretation. T.Y.: conception and design and administrative support; T.Y.: financial support and administrative support.

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Abbreviations Used
ABC2 = ATP binding cassette subfamily G member 2
ANOVA = analysis of variance
BBB = blood–brain barrier
bFGF = basic fibroblast growth factor
BMEC = brain microvascular endothelial cell
CNS = central nervous system
CSF = cerebrospinal fluid
DAPI = 4’,6-diamidino-2-phenylindole
DMSO = dimethyl sulfoxide
DPBS = Dulbecco’s phosphate-buffered saline
ECM = endothelial cell medium
ES cell = embryonic stem cell
Glut-1 = glucose transporter 1
HBSS = Hank’s Balanced Salt Solution
hiBBB = human iPSC-derived BBB
HPSCs = hematopoietic stem cells
HUVEC = human umbilical cord blood cell
iBMEC = iPSC-derived BMEC
ICC = immunocytochemistry
iPScs = induced pluripotent stem cells
LC/MS = liquid chromatography–mass spectrometry
LC/MSMS = liquid chromatography-tandem mass spectrometry
MRP-1 = multidrug resistance-associated protein 1
MW = molecular weight
OCT1 = organic cation transporter 1
PAMPA = parallel artificial membrane permeability assay
pBMEC = primary BMEC
P-gp = P-glycoprotein
PPP = platelet poor plasma
RA = retinoic acid
SEM = standard error of the mean
SLC7A5 = solute carrier family 7 member 5
TJ = tight junctions
Ulex = Ulex europaeus
UM = unconditioned medium
vWF = Von Willebrand factor

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