Supplementary data

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Functional investigation of solute carrier family 35, member F2 (SLC35F2) in three cellular models of the primate blood–brain barrier

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Supplementary Methods

Construction of HEK293 cells stably expressing HA tagged hSLC35F2 (hSLC35F2-HEK293) and mSLC35F2 (mSLC35F2-HEK293)

Based on the nucleotide sequence (GenBank accession no. hSLC35F2: NM_017515, mSLC35F2 : NM_028060), the cDNA encoding a full open-reading frame of hSLC35F2 and mSlc35f2 with HA tags at their 3′ ends were cloned from HeLa cells and mouse kidney cDNA using polymerase chain reaction (PCR). The cDNAs were subcloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) and transfected into HEK293 cells using polyethylenimine “Max” (molecular weight 40,000) and Opti-MEM (Polysciences, Warrington, PA, USA) according to the manufacturer’s protocol. The transfectants were selected after culture in the presence of G418 (800 μg/mL), and maintained at 37 °C in 5% CO_2 and 95% humidity in Dulbecco’s modified Eagle’s medium (low glucose; Invitrogen) supplemented with 10% fetal bovine serum and G418 (400 μg/mL).

Construction of SLC35F2 knockout PC-3 cells

hSLC35F2-targeted sgRNA was ligated into the lentiCRISPR v.2 plasmid (Addgene, Watertown, MA, USA) and the plasmid containing GFP-targeted sgRNA was employed as control. Lentiviral vectors with these plasmids were constructed by HEK293T cells as follows; Day 1: lentiCRISPR v.2 plasmid was cotransfected with Vsvg and Pax (lentivirus envelope and packaging plasmids) to semi-confluent HEK293T cells. Day 3: the medium was replaced by one containing 10 μM forskolin. Day 6: the supernatant was collected after filtration by 0.45 μm filter, 4× polyethylene glycol (PEG) solution (32% PEG-6000, 400 mM NaCl, and 40 mM HEPES) was added, and the solution was preserved at 4 °C for continuous rotation overnight. Day 7: the solution was centrifuged at 2,080 *g* for 1 h at 4 °C. After aspirating the supernatant, the pellet was suspended with cell culture medium containing 10 μg/mL polybrene (COSMO BIO co., Ltd., Tokyo, Japan). The medium containing lentiviral vector was exposed to PC-3 cells or IMR90-C4 for 24 h. Afterward, the

infected cells were selected by puromycin (1 μg/mL) for 2 weeks. hSLC35F2 knockout PC-3 cell lines were constructed as cell populations and not as cell clones. hSLC35F2 knockout IMR90-C4 was cloned with frameshift mutation in the SLC35F2 genome.

***In situ* mice brain perfusion test**

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The right common carotid artery was catheterized with a SP8 (Natsume Seisakusho, Osaka, Japan) or polyethylene tubing (PE-10; Becton Dickinson, Franklin Lakes, NJ, USA) filled and mounted on a 27-gauge needle (Terumo Corporation, Tokyo, Japan). Before insertion of the catheter, the common carotid artery and internal carotid artery were ligated caudally. During surgery, the body temperature was maintained by placing the mouse on a heated plate (Natsume Seisakusho). The syringe containing the perfusion fluid was placed in an infusion pump (Harvard Apparatus Syringe Infusion Pump, Harvard Apparatus, South Natick, MA, USA) and connected to the catheter. The thorax of the animal was opened and the heart was cut before perfusion. Perfusion was then started immediately at a flow rate of 1 mL/min. The perfusion fluid consisted of Krebs–Henseleit bicarbonate buffer (25 mM $NaHCO_3$, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1.2 mM $CaCl_2$, and 10 mM D-glucose). The perfusion fluid was bubbled for 30 min with 95% O_2 and 5% CO_2 for pH control (7.4) and warmed to 37 °C in a water bath. At 1 min after starting the perfusion, the mice were decapitated to terminate the perfusion. The brain was then removed, weighed and the right cerebrum was preserved at −20 °C. These specimens were stored at −20 °C until subjected to LC-MS/MS analysis.

The apparent volume of brain distribution (V_{brain}) was calculated using the following equation:

$$V_{brain} = \frac{X_{tot}}{C_{perf}}$$

where X_{tot} is the amount of test compound in the right cerebrum per gram of the right cerebrum, and C_{perf} is the concentration of test compound in the perfusate.

Supplementary Table 1. Primers and oligos sequences.

Primers/Oligos	Sequences
GFP sgRNA sense	CACCGGAGCTGGACGGCGACGTAAA
GFP sgRNA antisense	AAACTTTACGTCGCCGTCCAGCTCC
SLC35F2 sgRNA sense	CACCGAGTGCCACTTCCGTCAACCT
SLC35F2 sgRNA antisense	AAACAGGTTGACGGAAGTGGCACTC
mkSLC35F2 PCR forward	ATTGCTTTGGGATTCCTGTG
mkSLC35F2 PCR reverse	CCATGGTTCCTACACCCAAC

Supplementary Table 2. Analytical conditions for test compounds.

➤ Mass spectrometry: QTRAP5500

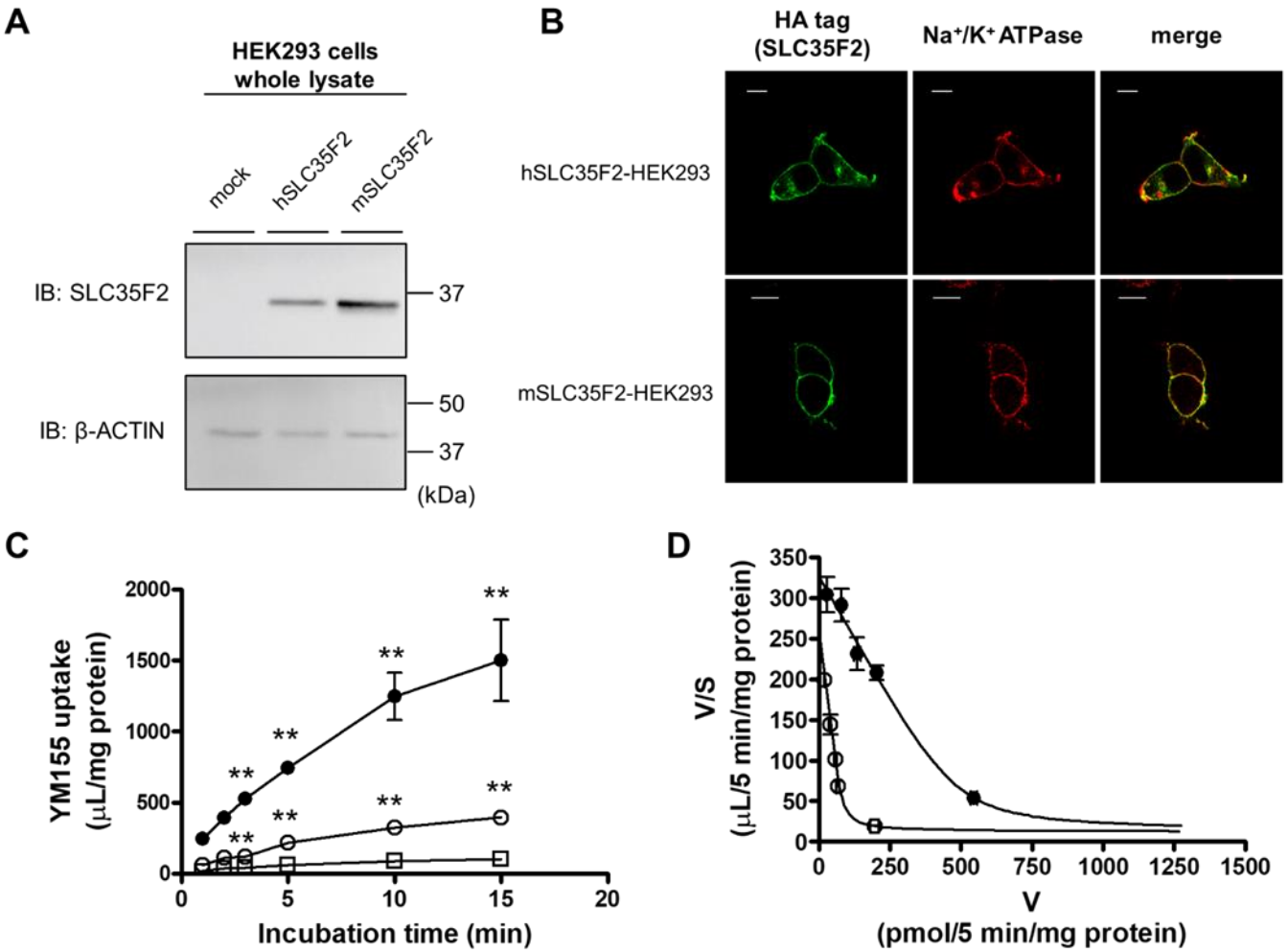
➤ Flow rate: 0.4 mL/min

Compounds	Mass-to-charge	Ion mode	Column	Mobile phase		Gradient condition
				A	B	(B conc. %)
Sulpiride	342.1→112.1	Positive	Atlantis T3 (3 μm, 2.1 mm × 50 mm; Waters, Tokyo, Japan)	0.1% formic acid in water	0.1% formic acid in acetonitrile	0 min; 3% 0.5 min; 3% 4.5 min; 90% 5 min; 90% 5.01 min; 3% 6 min; 3%
Pitavastatin	422.0→290.1					
Amantadine	152.1→107.2					
Memantine	180.0→107.1					
Pindolol	249.3→116.1					
Ym155	363.4→305.0					
Diazepam	285.2→193.0					
Diphenhydramine	256.2→167.2					
Pyrilamine	286.1→121.2					
Varenicline	212.2→169.1					
Amantadine	152.1→107.2					
Memantine	180.0→107.1					
Pindolol	249.3→116.1					
Pyrimethamine	249.1→177.0					
Procainamide	236.3→163.1					
Quinidine	325.1→184.2					
Dantrolene	313.0→214.0	Negative				
Clonidine	230.0→44.2	Positive	PC-HILIC S3 (3 μm, 2.0 mm × 150 mm Osaka-soda, Osaka, Japan)	10 mM ammonium acetate/0.1% formic acid/20% acetonitrile	10 mM ammonium acetate/0.1% formic acid/95% acetonitrile	0 min; 75% 4 min; 75%
Cimetidine	253.1→159.2					
Ranitidine	315.1→176.2					
Famotidine	338.3→305.0					
Nicotine	163.0→130.1					

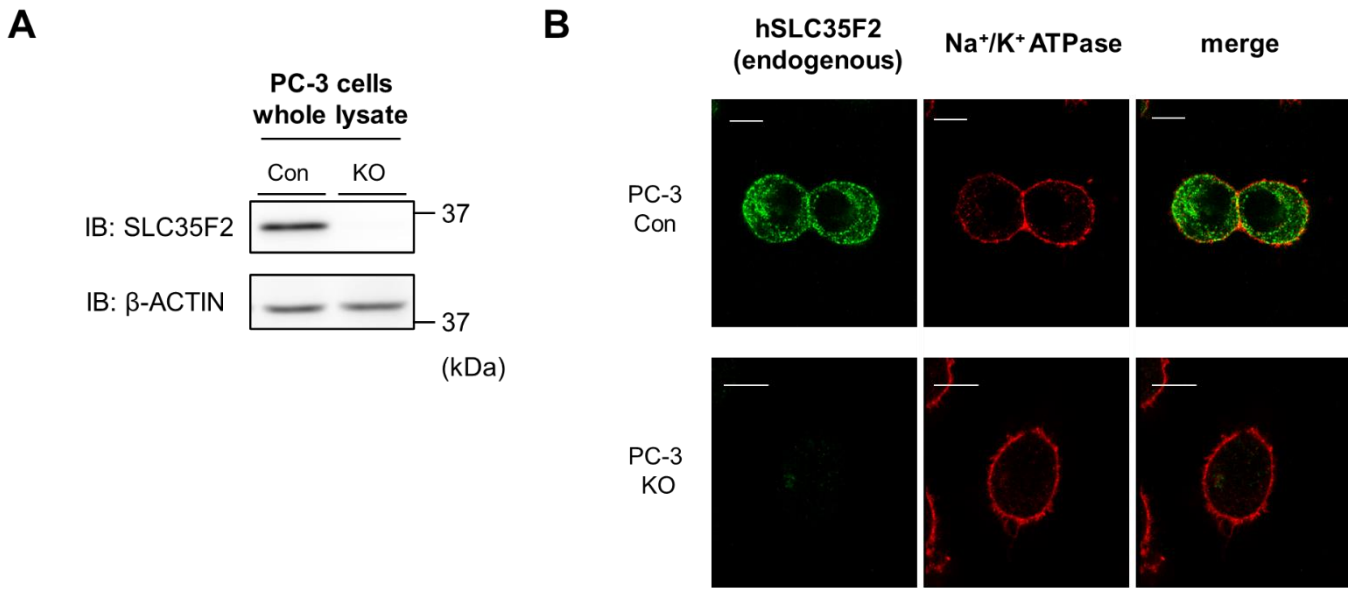
Supplementary Table 3. Batch differences in the quality of monkey BMECs (MBT-24H).

Lot No.	Lucifer yellow transport amount (pmol)	Quinidine B-to-A/A-to-B ratio	YM155 A-to-B control/famotidine ratio
24E50	24.6	4.87	1.78
2C003	N.D.	0.829	0.995
2C180	N.D.	1.55	1.09
2C251	119	2.40	1.15

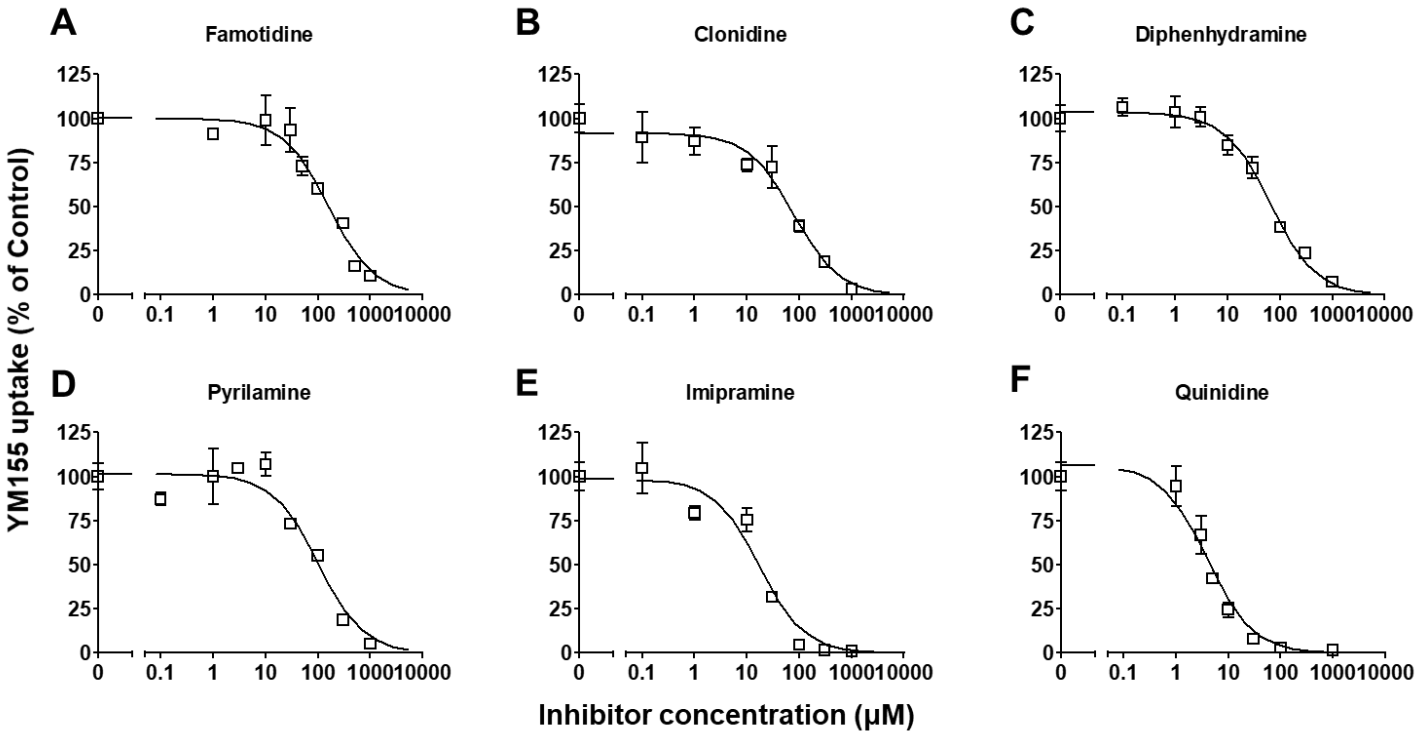
Four batches of monkey BMECs (MBT-24H) were compared. Lucifer yellow transport represents the average of A-to-B transport amount of lucifer yellow after 4 h incubation. The quinidine B-to-A/A-to-B ratio represents the ratio of B-to-A and A-to-B quinidine transport amount after 4 h incubation. The YM155 A-to-B control/famotidine ratio represents the ratio of control and 1mM famotidine-treated condition of A-to-B YM155 transport amount after 4 h incubation. The detailed test conditions were same as Figure 3. N.D. indicates no data. The experiments were conducted once by each lot.


A
HEK293 cells
whole lysate
mock
hSLC35F2
mSLC35F2
IB: SLC35F2
37
IB: β-ACTIN
50
37
(kDa)
B
HA tag
(SLC35F2)
Na+/K+ATPase
merge
hSLC35F2-HEK293
mSLC35F2-HEK293
C
YM155 uptake
(μL/mg protein)
2000
1500
1000
500
0
0
5
10
15
20
Incubation time (min)
**
D
V/S
(μL/5 min/mg protein)
350
300
250
200
150
100
50
0
0
250
500
750
1000
1250
1500
V
(pmol/5 min/mg protein)

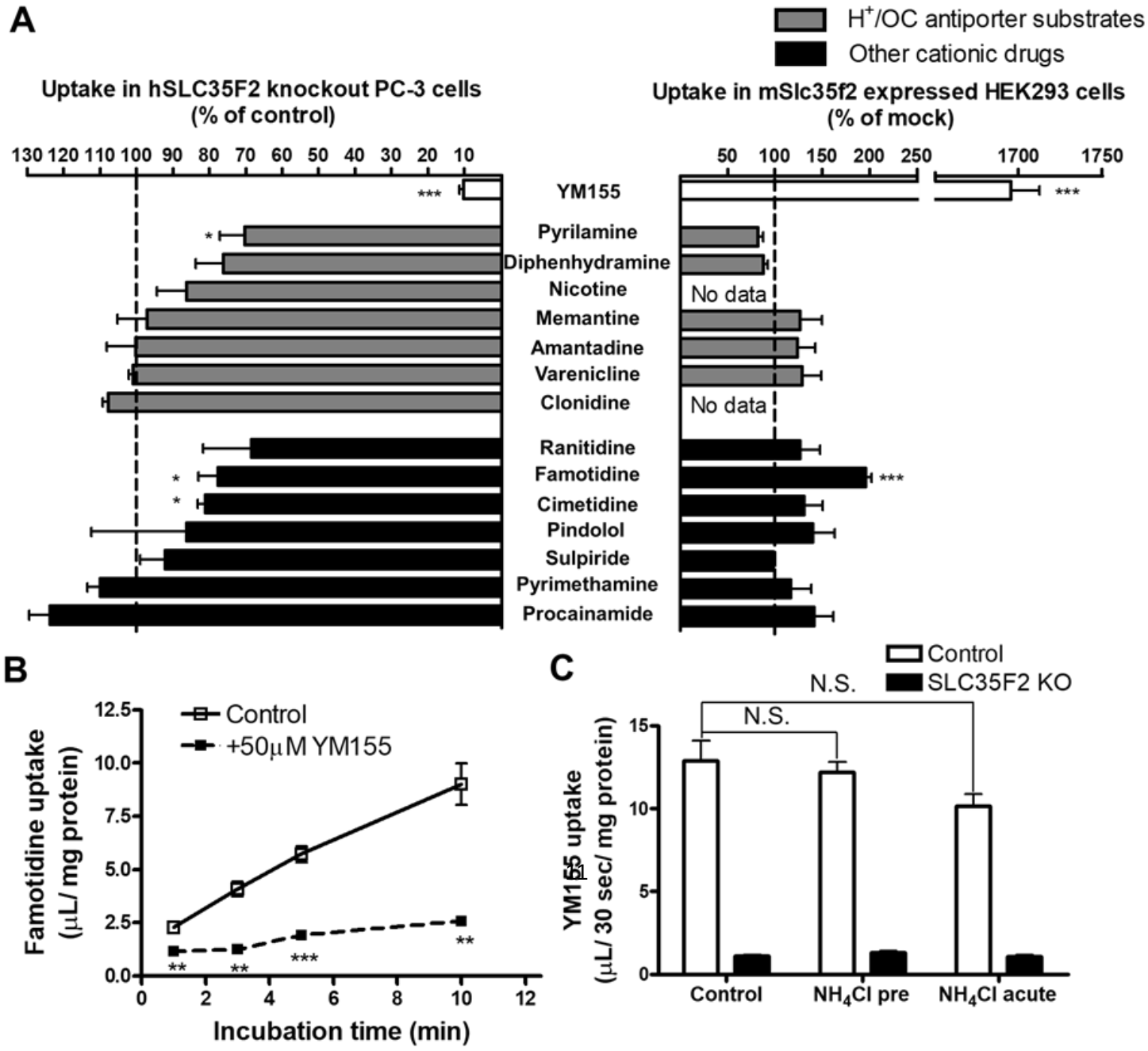
Supplementary Figure 1. YM155 uptake in HEK293 cells stably expressing hSLC35F2 and mSLC35F2. (A) Protein expression levels of hSLC35F2 and mSLC35F2 in HEK293 cells. Mock, hSLC35F2, and mSLC35F2 represent whole lysates of mock, hSLC35F2-HEK293, and mSLC35F2-HEK293, respectively. (B) Intracellular localization of SLC35F2 in hSLC35F2-HEK293 and mSLC35F2-HEK293. Green and red fluorescence represent HA tag and Na^+/K^+ ATPase (basolateral membrane marker), respectively. Scale bars represent 10 μm in all images. (C) Time courses of YM155 (0.1 μM) uptake in mock (open squares), hSLC35F2-HEK293 (open circles) and mSLC35F2-HEK293 (closed circles). The error bars represent the standard error of mean (n = 2–3, mean ± SE). (D) Eadie–Hofstee plots for 5 min YM155 uptake in hSLC35F2-HEK293 (open circles) and mSLC35F2-HEK293 (closed circles). The lines represent fitting curves and fitted equation were shown in the Methods. The plots were accounted for by a saturable and nonsaturable component. The fitted $K_{m,\ app}$, $V_{max,\ app}$, and PS_{diff} were calculated as shown in Table 1. The error bars represent the standard error of mean (n = 3, mean ± SE). $^{**}p < 0.01$, $^{***}p < 0.001$ vs mock cells. The experiments were repeated at least twice.



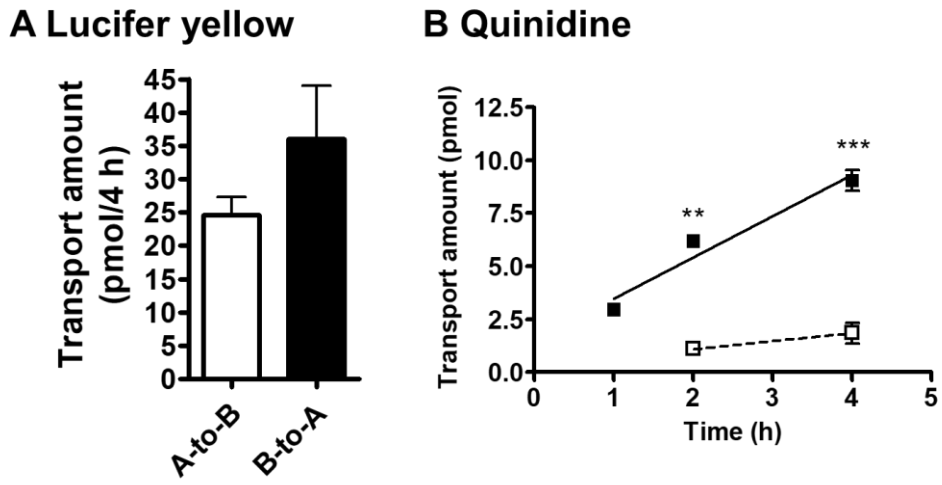
Supplementary Figure 2. SLC35F2 expression and localization in control and SLC35F2 knockout PC-3 cells. (A) Protein expression level of SLC35F2. Con and KO represent the specimens of control and SLC35F2 knockout PC-3 cells, respectively. (B) Intracellular localization of SLC35F2 in PC-3 cells. Endogenous SLC35F2 signals were stained with green. Na^+/K^+ ATPase, cell surface marker, signals were stained with red. Scale bars represent 10 μM in all images. The experiments were repeated at least twice.



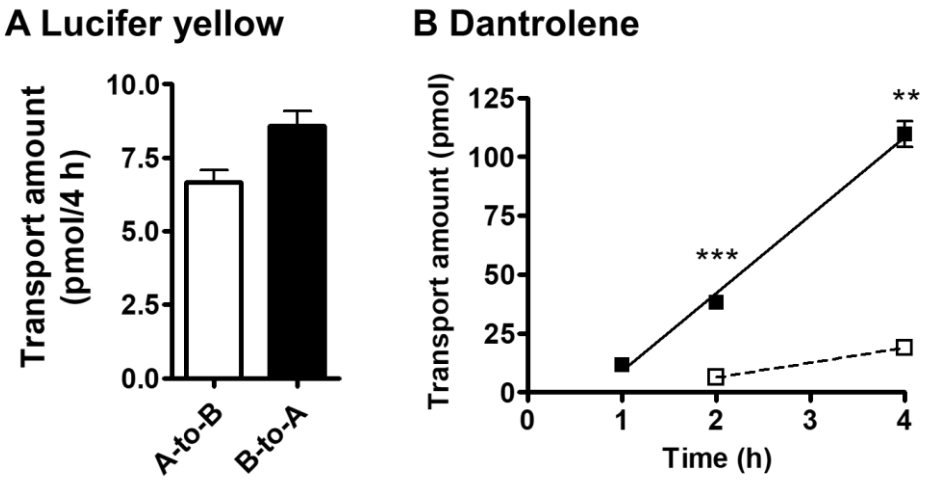
Supplementary Figure 3. Inhibitory effects of cationic drugs on YM155 uptake mediated by SLC35F2 in control PC-3 cells. YM155 uptake mediated by SLC35F2 was examined for 5 min in the presence and absence of (A) famotidine, (B) clonidine, (C) diphenhydramine, (D) pyrilamine, (E) imipramine, and (F) quinidine. Solid lines represent fitted curves for the inhibition of YM155 uptake by the inhibitors that were obtained by nonlinear least-squares regression analysis. The calculated half-maximal inhibitory concentration values were shown in Table 3. The error bars represent the standard error of mean (n = 3, mean ± SE). The experiments were repeated at least twice.


A
H+/OC antiporter substrates
Other cationic drugs
Uptake in hSLC35F2 knockout PC-3 cells
(% of control)
Uptake in mSlc35f2 expressed HEK293 cells
(% of mock)
130 120 110 100 90 80 70 60 50 40 30 20 10
50 100 150 200 250 1700 1750
YM155
Pyrilamine
Diphenhydramine
Nicotine
Memantine
Amantadine
Varenicline
Clonidine
Ranitidine
Famotidine
Cimetidine
Pindolol
Sulpiride
Pyrimethamine
Procainamide
No data
No data
B
Control
+50μM YM155
Famotidine uptake
(μL/ mg protein)
Incubation time (min)
C
Control
SLC35F2 KO
N.S.
N.S.
YM155 uptake
(μL/ 30 sec/ mg protein)
Control
NH4Cl pre
NH4Cl acute

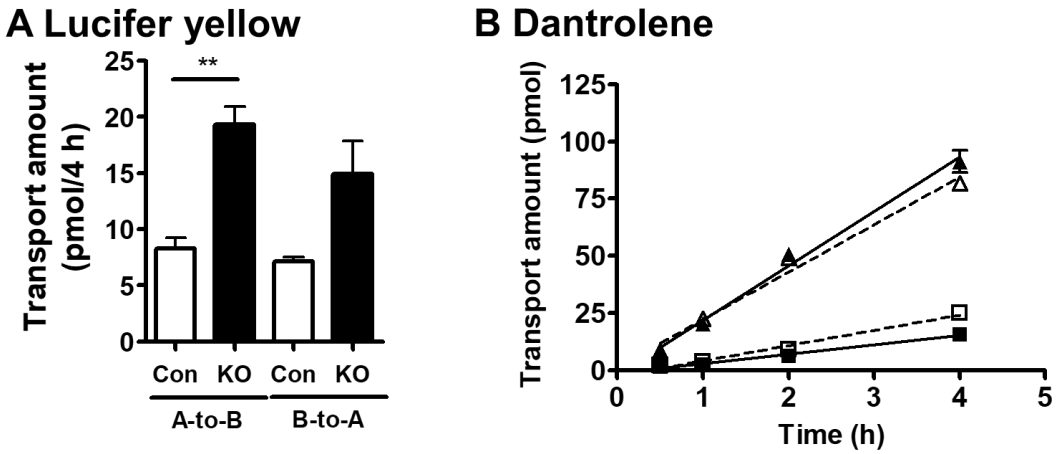
Supplementary Figure 4. Investigation of SLC35F2 substrate drugs and driving force. (A) Various drug uptake in hSLC35F2 knockout PC-3 cells for 5 min (left) and mSLC35F2-HEK293 (right) for 10 min. The values were expressed as percentages of uptake rates in control PC-3 and mock HEK293, respectively. (B) 1 μM famotidine uptake in mSLC35F2-HEK293 with (closed symbols) or without (open symbols) 50 μM YM155. Effects of proton gradient on YM155 uptake in PC-3 cells. To study the proton dependency of the uptake, we employed NH_4Cl as the reagent in constructing a proton gradient between the inside and outside of the cells. Cells were preincubated with 30 mM NH_4Cl for 10 min and sequentially removed for acidification of pH_i (NH_4Cl pre). The uptake was measured in the presence of 30 mM NH_4Cl to elevate pH_i (NH_4Cl acute). Open and closed bars represent values in control and SLC35F2 knockout PC-3 cells, respectively. The error bars represent the standard error of mean (n = 3, mean ± SE). N.S. indicates $p \geq 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control uptake. The experiments were repeated at least twice.



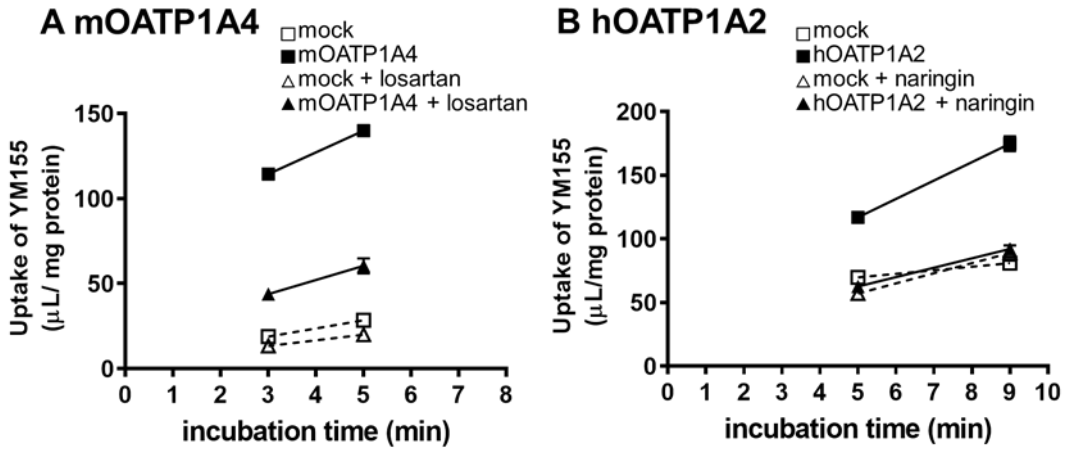
Supplementary Figure 5. Quality validation of monkey BMECs (related to Figure 3). (A) Lucifer yellow (10 μM) was permeable across the monkey BMECs after 4 h incubation. Thus, lucifer yellow was employed as a paracellular transport marker. The permeability was low enough to conduct following permeability tests evaluating transcellular transport. (B) Quinidine (0.1 μM) was permeable across the monkey BMECs. Quinidine was employed as a marker for P-gp transport function. The open and closed squares represent the permeable amounts in the A-to-B and B-to-A directions, respectively. The error bars represent the standard error of mean (n = 3, mean ± SE). ** $p < 0.01$, *** $p < 0.001$ vs permeable amount in the A-to-B direction. The experiments were conducted once in this lot (24E50).



Supplementary Figure 6. Quality validation of hiPS-BMECs (related to Figure 4). (A) Lucifer yellow (10 μM) was permeable across the hiPS-BMECs after 4 h incubation. Lucifer yellow was employed as a paracellular transport marker. (B) Dantrolene (0.1 μM) was permeable across the hiPS-BMECs. Dantrolene was employed as a marker of BCRP transport function. The open and closed squares represent the permeable amount in the A-to-B and B-to-A directions, respectively. The error bars represent the standard error of mean (n = 3, mean ± SE). ** $p < 0.01$, *** $p < 0.001$ vs control value of A-to-B direction. The experiments were repeated at least twice.



Supplementary Figure 7. Quality validation of SLC35F2 KO hiPS-BMECs (related to Figure 5). (A) Lucifer yellow (10 μM) was permeable across the cells after 4 h incubation. (B) Dantrolene (0.1 μM) was permeable on the A-to-B transport across control hiPS-BMECs (open squares) and SLC35F2 KO hiPS-BMECs (closed squares) and on the B-to-A transport across control hiPS-BMECs (open triangles) and SLC35F2 KO hiPS-BMECs (closed triangles), respectively. The error bars represent the standard error of mean (n = 4, mean ± SE). ***p* < 0.01 vs A-to-B transport in control hiPS-BMECs. The experiments were repeated at least twice.



Supplementary Figure 8. YM155 uptake mediated by mouse OATP1A4 and human OATP1A2 (related to Figure 6). (A) YM155 uptake test in mock or HEK293 stably expressing mouse OATP1A4 (mOATP1A4) with or without 100 μM losartan. Open squares, open triangles, closed squares, and closed triangles represent mock, mock with losartan, mOATP1A4, and mOATP1A4 with losartan, respectively. (B) YM155 uptake test in mock or HEK293 transiently expressing human OATP1A2 (hOATP1A2) with or without 100 μM naringin. Open squares, open triangles, closed squares, and closed triangles represent mock, mock with losartan, hOATP1A2, and hOATP1A2 with naringin, respectively. The error bars represent the standard error of mean (n = 3, mean ± SE). The experiments were repeated at least twice.