Improvement of protein expression profile in three-dimensional renal proximal tubular epithelial cell spheroids selected based on OAT1 gene expression: a potential in vitro tool for evaluating human renal proximal tubular toxicity and drug disposition

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3D RPTEC spheroids: an improved in vitro tool

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d) Abbreviations:

2D, two dimensional
3D, three dimensional
RPTEC, renal proximal tubular epithelial cells
OAT, organic anion transporter
OCT, organic cation transporter
MATE, multidrug and toxin extrusion
SLC, solute carrier
ABC, ATP-binding cassette
PM, plasma membrane
TL, total lysate

2. Abstract (247 words)
The proximal tubule plays an important role in the kidney and is a major site of drug interaction and toxicity. Analysis of kidney toxicity via in vitro assays is challenging, because only a few assays that reflect functions of drug transporters in renal proximal tubular epithelial cells (RPTECs) are available. In this study, we aimed to develop a simple and reproducible method for culturing RPTECs by monitoring organic anion transporter 1 (OAT1) as a selection marker. Culturing RPTECs in spherical cellular aggregates increased OAT1 protein expression, which was low in the conventional 2D culture, to a level similar to that in human renal cortices. By proteome analysis, it was revealed that the expression of representative two proximal tubule markers was maintained and 3D spheroid culture improved the protein expression of approximately 7% of the 139 transporter proteins detected, and the expression of 2.3% of the 4,800 proteins detected increased by approximately 5-fold that in human renal cortices. Furthermore, the expression levels of approximately 4,800 proteins in 3D RPTEC spheroids (for 12 days) were maintained for over 20 days. Cisplatin and adefovir exhibited transporter-dependent ATP decreases in 3D RPTEC spheroids. These results indicate that the 3D RPTEC spheroids developed by monitoring OAT1 gene expression are a simple and reproducible in vitro experimental system with improved gene and protein expressions compared to 2D RPTECs and were more similar to that in human kidney cortices. Therefore, it can potentially be used for evaluating human renal proximal tubular toxicity and drug disposition.

3. Significance statement
We developed a simple and reproducible spheroidal culture method with acceptable throughput using commercially available renal proximal tubule epithelial cells (RPTEC) by monitoring OAT1 gene expression. RPTECs cultured using this new method showed improved mRNA/protein expression profiles to those in 2D RPTECs and were more similar to those of human kidney cortices. This study provides a potential in vitro proximal tubule system for pharmacokinetic and toxicological evaluations during drug development.

4. Visual abstract

Not applicable
5. Introduction (656 words)

The proximal tubule of the nephron—the primary site for the reabsorption and secretion of endogenous and exogenous substances—has a biotransformation capacity. Numerous enzymes are involved in the metabolism and clearance of endogenous and exogenous compounds in the kidney, including cytochrome P450 (CYP) and non-CYP enzymes, such as uridine-diphosphate-glucuronosyltransferases (UGTs), esterases, glutathione-S-transferases, and sulfotransferases (Bajaj et al., 2018). Transporters expressed on the basal membrane transfer xenobiotics from the systemic circulation to proximal tubular cells. Subsequently xenobiotics are secreted into urine via transporters expressed on the brush border membrane of the proximal tubular cells, or vice versa to be reabsorbed from the filtrate.

Recently, the roles of tubular apical and basolateral transporters in eliminating and reabsorbing xenobiotics and endogenous substrates were elucidated (Morrissey et al., 2013; Nigam et al., 2015; Miners et al., 2017). Examples of endogenous transporters include urate transporter 1 (URAT1; SLC22A12) and glucose transporter 9 (GLUT9; SLC2A9) as apical and basolateral urate transporters, respectively, SLC34A1/3 as an apical phosphate transporter (Segawa et al., 2015), sodium coupled monocarboxylate transporter 1/2 (SMCT1/2; SLC5A8/12) as an apical lactate transporter, sodium-glucose co-transporter 1/2 (SGLT1/2; SLC5A1/2) and GLUT2 (SLC2A2) as apical and basolateral glucose transporters, respectively, organic cation/carnitine transporter 1 (OCTN1; SLC22A4) as an apical ergothioneine transporter, OCTN2 (SLC22A5) as an apical carnitine transporter, and peptide transporter 1 (PEPT1; SLC15A1) as an apical peptide transporter. Regarding the drug transporters in the kidneys, basolateral organic anion transporters 1 and 3 (OAT1 and OAT3, respectively) accept organic anions as substrates. OAT1 and OAT3 have overlapping substrate specificities; however, OAT1 recognizes organic anions with relatively smaller molecular weights than OAT3 (Hasegawa et al., 2002, 2003; Sweet et al., 2003; Deguchi et
al., 2004; Early et al., 2006; Nozaki et al., 2007). The organic cation transporter 2 (OCT2) on
the basolateral side and the multidrug and toxin extrusion 1/2-K (MATE1/2-K) at the brush
border of proximal tubular cells play important roles in the renal secretion of organic cations
(Hillgren et al., 2013). Regulatory bodies such as the US Food and Drug Administration,
European Medical Agency and Pharmaceuticals and Medical Devices Agency, recommend
that pharmaceutical industries evaluate the interactions between candidate drugs and drug
transporters such as OAT1, OAT3, OCT2, MATE1 and MATE2-K.

Typically, nephrotoxicity, which is detected only in the late stages of drug development,
accounts for 2% of drug attrition in preclinical studies and 19% in phase 3 trials (Jang et al.,
2013). These problems are associated with increased risks for patients and subjects enrolled in
clinical trials as well as substantial costs for the health care system and the pharmaceutical
industry. A major problem is the lack of preclinical models that offer high predictability. The
predictability of animal models is compromised by interspecies variability. Drug-drug
interactions in the kidney and the effects of the drug-induced tubular injury have been
investigated using various systems, such as kidney slices, cell isolation methods, cultures of
established kidney cell lines and human kidney epithelial cell lines (Tiong et al., 2014).
Nakanishi et al., (2011) reported that mRNA expression of multiple solute carrier (SLC)
transporters in rat proximal tubular cells decreased dramatically when cultured using the
two-dimensional (2D) method. Several cell systems, such as LLC-PK1 cell line, maintain the
transport function of organic cations; however, only a limited number of cell systems have
sufficient OAT transporter functions (Hauwaert et al., 2014). To overcome the low OAT
functionality in cell systems, recently Nieskens et al., (2016) developed conditionally
immortalized human proximal tubule cells with OAT1 or OAT3 protein expression by
transfecting cDNA. However, cell models of RPTEC with sufficient drug transporter
functions including OAT1/3 and OCT2, have not yet been established.
In this study, we hypothesized that the expression of other genes and proteins could be improved along with changing the OAT1 gene expression. To test the hypothesis, we developed a three-dimensional (3D) spheroid culture method for commercial RPTECs by monitoring OAT1 mRNA expression as a selection marker, characterized mRNA and protein expression, transporter functions and cellular toxicity in 3D RPTEC spheroids and discuss the usefulness of 3D RPTEC spheroids for toxicity and pharmacokinetic analyses during drug development.

6. Materials and Methods

Chemicals and reagents

The RPTECs were purchased from LONZA (Walkersville, MD). Unlabeled cimetidine, digoxin, and furosemide were purchased from Sigma-Aldrich (St. Louis, MO). Metformin hydrochloride, probenecid and cisplatin were purchased from Fujifilm Wako (Osaka, Japan). Pyrimethamine was purchased from MP Biomedicals (Irvine, CA) and zosuquidar was purchased from Cellagen Technology (San Diego, CA). Adefovir was purchased from Cayman Chemical Company (Ann Arbor, MI). [3H]adeovir was purchased from Moravek Inc. (Brea, CA). All other chemicals and reagents were commercially available and of analytical grade.

Human renal cortex

Frozen human renal cortex was purchased from Reprocell Japan (Yokohama, Japan). Information on the tissue donors is presented in Table 1.

Cell culture of RPTEC

RPTEC maintenance culture

RPTECs (Lonza, Walkersville, MD, Item No. CC-2553) were used in this study. RPTECs
(passage 3) were maintained in renal epithelial cell growth medium (REGM; Lonza, Walkersville, MD) supplemented with 0.5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO2. The culture medium was replaced every two days until the cells reached confluence.

2D RPTEC

RPTECs (passage 3) were washed with phosphate-buffered saline (PBS), and treated with Accutase (Innovative Cell Technologies, San Diego, CA) for a few minutes at room temperature. Cell suspensions were seeded onto a cell culture plate (six well plate; Greiner AG) at a cell number of $3 \times 10^5$ cells/well. RPTECs (passage 4) were maintained in REGM supplemented with 0.5% FBS and culture until confluence. The culture medium was replaced every two days.

3D culture

RPTECs were washed with phosphate-buffered saline (PBS) and treated with Accutase (Innovative Cell Technologies, San Diego, CA) for a few minutes at room temperature. The cells were suspended at $1 \times 10^4$/mL in REGM. The cell suspension was plated into the wells of a V-bottom ultra-low attachment 96-well plate (PrimeSurface; Sumitomo Bakelite, Tokyo). In each well, the cell suspension formed spherical cellular aggregates of RPTEC (3D RPTEC; 1,000 cells/well). 3D RPTEC spheroids were maintained at 37°C in a humidified atmosphere with 5% CO2 in REGM, and the medium was replaced every 2–3 days.

Gene expression analysis

2D RPTECs or 3D RPTEC spheroids were directly lysed with the RLT lysis buffer and total RNA was isolated using an RNeasy Mini Kit (Qiagen Inc., Germantown, MD, USA) according
to the manufacturer’s instructions. The total RNA from the biopsy-embedded optimal cutting temperature (O.C.T) compound (donor 1) was extracted using the RNeasy Mini Kit from Aproscience (Tokushima, Japan). Total RNA from snap-frozen autopsies (donors 2–5) was obtained from Reprocell (Yokohama, Japan). RNA quality was assessed using an Agilent TapeStation to determine the 28S/18S rRNA ratio. The cDNA was synthesized from the total RNA using QuantiTect Whole Transcriptome Kit (Qiagen, Hilden). Quantitative real-time PCR was performed using TB green Premix EX Taq II (Takara Bio, Shiga, Japan) and primer sets (OAT1: Forward 5′-CTGTATCCCACAATGATCCG-3′, Reverse 5′-GGCAGTCATGCTCACCAG-3′; GAPDH: Forward 5′- TTGACGCTGGGGCTGGCATT-3′, Reverse 5′- GTGCTCTTGCTGGGGCTGGT-3′) on a Thermal Cycler Dice Real-Time System Single (Takara Bio, Shiga). Expression levels were calculated using the comparative -ΔΔCt method and normalized to GAPDH as an endogenous control in the same sample. For microarray analysis, RNAs with an RNA integrity number (RIN) > 6.0, were hybridized to the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) at the Takara Bio according to manufacturer’s protocol. Expression data were analyzed using GeneSpring software (Agilent Technologies, Santa Clara, CA).

**Preparation of total lysates and plasma membrane fractions**

Frozen human renal cortices were washed with ice-cold PBS, then homogenized by BioMasher II (Nippi. Inc, Tokyo) in buffer A (0.15 M KCl, 0.1 M phosphate buffer pH 7.4 and 0.5 mM phenylmethylsulfonyl fluoride) before total lysate preparation. 2D RPTECs, 3D RPTEC spheroids and HEK293 cells were washed with ice-cold PBS and suspended in buffer A before total lysate preparation. Total lysates were prepared by ultrasonication for total 1.5 min (10 sec x 9 times) using Bioruptor (BM Equipment, Tokyo). Plasma membrane fractions were further enriched from the total lysate as described previously (Schaefer et al., 2018). Briefly, total
lysates were centrifuged at 10,700 × g for 20 min at 4°C, post-nuclear supernatants collected and sedimented twice at 100,000 × g for 60 min at 4°C. The resulting microsomal pellet was suspended in buffer B (0.25 M sucrose, 20 mM Tris-HCl, pH 7.4), layered on top of a 38% (w/v) sucrose solution and ultracentrifuged at 100,000 × g for 40 min at 4°C. The turbid interface fraction was recovered, suspended in buffer B, and sedimented at 100,000 × g for 60 min at 4°C. The plasma membrane fraction was obtained by suspending the pellet in MPEX PTS reagent B (GL Science, Tokyo). Protein concentration of total lysate and plasma membrane fraction was determined using the CBQCA Protein Quantitation kit (Thermo Fisher, Waltham, MA).

**Sample preparation for tandem mass tag (TMT)-based proteomics.**

Proteins in total lysates and plasma membrane fractions for liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics analysis were prepared using the single-pot solid-phase-enhanced sample preparation (SP3) method (Sielaff et al., 2017; Hughes et al., 2019) and labeled with the TMTpro reagent (Thermo Fisher Scientific, Waltham, MA) as previously described (Ohtsu et al., 2022). In brief, 9 μg of samples were dissolved in 100 mM triethylammonium bicarbonate (TEAB) (Thermo Fisher Scientific) and 150 mM NaCl solution with MPEX PTS reagent B (GL Science, Tokyo, Japan), and then reduced with tris-(2-carboxyethyl) phosphine (TCEP) (Thermo Fisher Scientific) followed by alkylation with iodoacetamide (Thermo Fisher Scientific). After protein purification using Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles (SP3 beads) (GE Healthcare, Chicago, IL), the samples were digested with trypsin/LysC mix (Promega, Madison, WI). The total concentration of the purified peptides was determined using a Fluorometric Peptide Assay Kit (Thermo Fisher Scientific). Then the peptides were labeled with TMTpro reagent, fractionated (into 12 fractions) with Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo
Fisher Scientific), and dried.

**Nano-LC-MS/MS of TMT-labeled peptides.**

After the reconstitution with 0.1% formic acid/2% acetonitrile (eluent A), an aliquot of the peptide mixtures (2 μg/10 μL) was injected into the LC-MS/MS system consisting of an UltiMate 3000 RSLCnano LC system coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). The peptides were loaded on a PepMap100 C18, 2 cm x 100 μm i.d., 5 μm trap column (Thermo Fisher Scientific) and subsequently, separated on Aurora Series UHPLC emitter columns (25 cm x 75 μm i.d., 1.6 μm; IonOpticks, Melbourne) heated to 45°C at a flow rate of 400 nL/min using a gradient of 2–24% eluent B (0.1% formic acid/100% acetonitrile) for 240 min, 24–32% eluent B for 30 min, and 32-95% eluent B for 10 min followed by 95% eluent B wash step for 10 min. The mass spectrometer was operated in multi-notch synchronous precursor selection (SPS) mode.

**Immunofluorescence microscopy**

3D RPTEC spheroids were fixed in methanol for 10 min at −20°C. Then, the spheroids were washed twice with PBS and incubated in a blocking solution (0.1% Triton X-100 and 5% donkey serum in PBS) for 60 min and then with rabbit polyclonal anti-P-gp antibody (Abcam, ab129450) and goat polyclonal anti-Na⁺/K⁺-ATPase antibody, N-15 (SantaCruz, SC-16041) at 4°C overnight. Immunoreactions were visualized by cell incubation with Anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific) for P-gp and Anti-goat Alexa Flour 647 (Thermo Fisher Scientific) for Na⁺/K⁺-ATPase. Finally, the 3D-RPTEC spheroids were mounted with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and observed using a Zeiss LSM 800 (Carl Zeiss AG, Oberkochen) and Zeiss C-Apochromat 63 × 1.2 W Correction Ring water immersion objective. All images were analyzed using the
LSM 800 software.

Transport experiments

Approximately 200 wells of 3D RPTEC spheroids cultured for 12-14 days were collected in one tube. The collected spheroids were washed with an assay buffer (pH 7.4), consisting of Hanks’ balanced salt solution (HBSS) supplemented with 15 mM HEPES, by repeating the following cycle twice; 1) addition of the assay buffer, 2) centrifugation at 100 × g for 3 min, and 3) aspiration of the supernatant. The pellet of spheroids was re-suspended in the assay buffer and then equilibrated for 15 min at 37°C. After centrifugation (100 × g, 3 min) and removal of the supernatant, uptake was initiated by adding an assay buffer containing either a transporter cocktail (10 μM metformin, 10 μM furosemide, 10 μM digoxin) or [3H]adefovir (80.6 nM) in the presence or absence of each transporter inhibitor. After the designated incubation time, the spheroids were centrifuged at 500 × g for 1 min. The concentrations of compounds in the assay were determined using the supernatant. Uptake was terminated by removing the supernatant, followed by the addition of ice-cold assay buffer (3 mL), centrifugation at 500 × g for 1 min, and removal of the supernatant. The cells were solubilized in NaOH for approximately 1 h at room temperature. The lysates were neutralized with HCl. The concentration of the drug in cell lysates was measured either by LC-MS analysis or with a liquid scintillation counter (AccuFLEX LSC-7200, Hitachi, Tokyo) after mixing with a scintillation cocktail (Clear-sol I, Nacalai Tesque, Inc., Kyoto). Protein concentrations were determined using the Lowry method (Lowry et al., 1951) or BCA Protein Assay Kit (Fujifilm-Wako Chemicals, Osaka) with bovine serum albumin as the protein standard.

LC-MS/MS analysis for drugs

Cell lysate (20 μL) was mixed with 60 μL of internal standard solution (acetonitrile with 0.1%
formic acid containing 200 nmol/L Hesperetin) followed by centrifuging at 10,000 × g for 5 min. The supernatant (45 μL) was mixed with 10 mmol/L ammonium formate (45 μL). After the sample preparation, an aliquot of the samples (5 μL) was injected into the LC-MS/MS system consisting of an ExionLC™ AD LC system coupled to a QTRAP5500 mass spectrometer (AB SCIEX, Framingham, MA). The samples were separated on Kinetex column, 2.6 μm, F5, 100 Å, 2.1 mm I.D.×100 mm (Phenomenex, Torrance, CA) heated to 40°C with a constant flow rate of 0.4 mL/min of mobile phases (A) 5 mmol/L ammonium formate and (B) acetonitrile and a gradient elution program. The program started with 10% eluent B and increased to 95% at 2.6 min followed by a wash step with 95% eluent B for 1 min before equilibration for 1.4 min with 10% eluent B. The total LC run time was 5 min. Metformin, furosemide, and digoxin were monitored using multiple reaction monitoring (MRM). The ionization conditions and m/z transitions are listed in (Supplemental Table 1). The limits of detection for metformin, furosemide, and digoxin were 1 nmol/L. The mean accuracy of the intra- and inter-run Quality Control samples were within 80 -120% (data not shown).

**In vitro ATP assay**

3D RPTEC spheroids (on days 10-12) in a V-bottom ultra-low attachment 96-well plate (PrimeSurface®; Sumitomo Bakelite, Tokyo) were exposed with respective concentrations of cisplatin and adefovir in REGM supplemented with 0.5% FBS. The exposure medium was replaced on days 3 and 5. Intracellular ATP levels were assessed using CellTiter-Glo® 3D Cell Viability Assay (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, 100 μL of the reagent was plated into each well and incubated for 30 min at room temperature. Bioluminescence was measured using a Wallac ARVO MX 1420 luminometer (Perkin Elmer, Waltham, MA). The ATP concentration was determined using an ATP standard curve.
Data analysis

Uptake experiment data analysis

Uptake clearance was calculated by normalizing the amount of compound inside the cells to that in the initial solution and the protein concentration in each tube using the following equation:

\[
Uptake\ CL = \frac{X_{cell}}{C_{buffer} \times \text{Protein}}
\]

where \(Uptake\ CL\) is the uptake clearance (μL/designated time point/mg), \(X_{cell}\) is the amount of compound in the cells (pmol/designated time/tube), and \(C_{buffer}\) is the concentration of the compound in the initial solution (pmol/μL). Uptake CL was normalized to the amount of total cellular protein (mg/tube).

Proteomics data analysis

Proteins were identified and quantified using Proteome Discoverer (version 2.4; Thermo Fisher Scientific). Briefly, the MS/MS spectra were searched against UniProtKB/Swiss-Prot human data downloaded in March 2020 using the SEQUEST HT search engine. Two missed cleavages were allowed, along with carbamidomethylation of cysteine as a fixed modification. Variable modifications included oxidation of methionine, deamidation of asparagine/glutamine, acetylation/methionine-loss/methionine-loss + acetylation of the protein N-terminus and phosphorylation of serine, tyrosine and threonine. Mass tolerance for precursor and fragment ions was 10 ppm, and 0.6 Da, respectively, and false discovery rates at the peptide and protein levels were less than 0.01. Relative protein abundances were calculated from the sum of the peptide ion abundances of unique and razor peptides which are peptides unique to a protein group and shared peptides in a protein group with more identified peptides, respectively. The peptide ion abundances were quantified using TMT reporter ion...
signal-to-noise ratios in MS3 scans, with a co-isolation threshold of 50 and an average reporter S/N threshold of 10. To normalize the quantitative data across TMT channels, the total peptide amount for each TMT channel was calculated and corrected for all abundance values in all other channels using a constant factor per channel. The normalized peptide abundances were then summed to calculate the protein abundances. To bridge the quantitative values across the two TMT16-plex experiments, all protein abundance values were scaled to control the channel average, which was set for a common sample over two-batch experiments. The calculated protein abundances were used for further analysis.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) or MS-Excel 2010 (Microsoft Corporation, USA). Data were presented as mean ± standard deviation (SD) for at least three independent experiments. Unpaired two-tailed Student’s *t*-test was used to compare datasets from independent groups of cells. Multiple comparisons were performed using one-way analysis of variance followed by Dunnett’s test. Data were considered statistically significant at <0.05. The 3D RPTEC spheroids/human cortex ratios of the adjusted geometric means of mRNA expression (geometric mean ratio, GMR) and their two-sided 90% confidence intervals (CIs) were computed using an analysis of variance on a logarithmic scale. For exploratory purposes, equivalences were evaluated when the GMR point estimate was 5-fold (≤20% or ≥500%), 3-fold (≤30% or ≥300%) and 2-fold (≤50% or ≥200%).

**Data availability**

and result files were deposited in the ProteomeXchange Consortium (http://www.proteomexchange.org/; PXID, PXD037535) via the jPOST partner repository (https://jpostdb.org; jpost ID, JPST001891) (jPOSTrepo., 2017).
7. Results

**OAT1 mRNA expression in 3D RPTEC spheroids**

When RPTECs were cultured in spherical cellular aggregates at a seeding cell number of 1,000 cells/well, OAT1 mRNA increased in accordance with the culture period (Fig. 1A). OAT1 mRNA showed the highest level at a seeding cell number of 1,000 cells/well and decreased at a seeding cell number greater than 1,000 cells/well (Fig. 1B). The size of the spheroid showed seeding cell number dependence; the size at a seeding cell number of 1,000 cells/well was approximately 271±19 µm (n=10; data not shown). Based on these results, a seeding cell number of 1,000 cells/well was chosen for further studies.

**mRNA expression in 2D RPTECs and 3D RPTEC spheroids in comparison to human renal cortical tissues**

The mRNA expression of the renal transporters, OCT2, OAT1, OAT3, MATE1 and MATE2-K recommended by regulatory authorities (US food and drug administration, 2020; European Medicines Agency 2012; Ministry of Health Labour and Welfare, 2018), in the 3D spheroid condition was compared with that in RPTECs cultured under 2D conditions. In 2D RPTECs even on day 12, the mRNA expression of the transporters was less than 27% of the average mRNA expression in human kidney cortical tissues from five donors (Fig. 2 A and C). With the exception of OAT3, gene expression in 3D RPTEC spheroids increased and was within the variability range of that of human kidney cortices (Fig. 2B and C).

Additionally, the mRNAs of 3,194 enzymes, 334 SLC transporters, and 39 ATP-binding cassette (ABC) transporters were compared among 2D RPTECs, 3D RPTEC spheroids, and human kidney cortices (Fig. 2 D-F). Approximately 90% of the mRNA of the detected 3,194 enzymes in 2D and 3D RPTECs at all culture periods was within the 5-fold difference of the average mRNA expression in human kidney cortices, although more
enzymes showed similar mRNA expression in 3D RPTECs at culture periods other than day 5. Approximately 80% of the detected ABC transporters when cultured in 2D on days 2 and 12 and in 3D on day 5 showed a difference within 5-fold of the average mRNA expression in the human kidney cortices. When RPTECs were cultured under 3D spheroid conditions for more than five days, the % of ABC transporters showing a difference within 5-fold increased to 90%. In 2D RPTECs, 73% and 25% of the detected SLC transporters on day 2 showed a difference within 5-fold and less than 20% of the average mRNA expression in human kidney cortices, respectively, and these values changed to 83% and 16%, respectively, on day 12. In 3D RPTEC spheroids, 82% and 17% of the detected SLC transporters on day 5 showed a difference within 5-fold and less than 20% of the average mRNA expression in human kidney cortices, respectively, and these values changed to 83% and 16%, respectively on day 12. The mRNA expression patterns of SLC transporters did not change largely beyond day 12. Next, we compared the mRNA expression profiles of 3D RPTEC spheroids from three different donors (days 12-14) with those of human kidney cortices from five different donors. The equivalence between 3D RPTEC spheroids and the human kidney cortex was investigated using GMR and their 90% CIs by applying 2-, 3-, and 5-fold differences (Table 2), because the coefficient of variation × 100 (CV%) of mRNA expression of the majority of the detected genes (16,985) in human kidney cortices was > 20 CV% (Supplemental Table 4). When a 5-fold difference was used as a benchmark, 92% of all genes, 87% of enzymes, 77% of SLC transporters and 44% of ABC transporters were judged to be statistically equivalent to those in the human kidney cortex. The percentage of genes decreased when low-fold differences (2- and 3-fold) were applied.

Protein expression in 2D RPTECs and 3D RPTEC spheroids in comparison to human renal cortical tissues

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Protein expression in the total lysate (TL) of 2D RPTECs, 3D RPTEC spheroids and human kidney cortices was investigated using label-based LC-MS/MS proteomics to examine whether the results based on mRNA, often used as a surrogate for protein expression, were observed in protein expression. A total of 4,848 proteins were quantified and approximately 90% of the detected proteins showed a 0.2- to 5-fold difference in the average protein expression in human kidney cortices; the same % was observed in the detected 1,642 enzymes and 21 ABC transporters (Table 3). In contrast, 70.5% and 25.9% of the 139 SLC transporters detected in 2D RPTECs showed a difference within 5-fold in average protein expression and less than 20% in the human kidney cortices, respectively. When RPTECs were cultured in 3D spheroid conditions, the % of proteins showing a 0.2- to 5-fold difference improved to 78.4%. The impact of the 3D spheroidal culture condition, selected by monitoring OAT1 gene expression, was further assessed (Supplemental Tables 2 and 3). When a 5-fold difference was used as the criterion for assessing the similarity between human kidney cortices and RPTECs, a total of 203 proteins showed similar protein expression to that in human kidney cortex, compared to 2D RPTECs. In contrast, 85 proteins showed diversified expression in 3D RPTEC spheroids, compared to 2D RPTECs. The protein expression of more than 20 transporters has been investigated using the total membrane fraction of human kidney cortices from 41 different donors by the LC-MS based quantitative targeted proteomics method (Prasad et al., 2016). However, it was difficult to compare the absolute expression levels between the two studies because TL and a different proteomics method were used in our study. However, it was possible to evaluate the appropriateness of the human cortical samples used in our study by comparing the variability in the protein expression of transporters between the two studies. The CV% of protein expression of the 11 transporters (OAT1, OAT3, OAT4, OCT2, OCTN1, OCTN2, SGLT2, P-gp, ABCC2, ABCC4, and MATE1) was analyzed in both studies. Except for OCTN1 and MRP2, the transporters showed similar CV% within a
1.6-fold difference. The CV% for OCTN1 and MRP4 differed by 1.8 and 5.0, respectively. Since 5-6 outliers were observed in 41 samples for OCTN1 and MRP4 in the study by Prasad et al., (2016), and showed larger variability, the CV% in our study is considered to be similar if outliers are omitted from the results of Prasad et al., (2016), indicating that the samples used in our study were valid (Fig. 3B-D).

**Protein expression of kidney markers and selected SLC and ABC transporters**

RPTECs are renal epithelial cells isolated from human proximal tubules. However, they may dedifferentiate during the culture process, and the original function of proximal tubular cells may not be maintained. The properties of the RPTECs were assessed based on the protein expression of podocalyxin (PODXL) and nephrin 1 (NPHS1) as representative glomerular markers, uromodulin (UMOD) and SLC12A1 as representative distal tubule markers, and aquaporin 1 (AQP1) and cadherin 6 (CDH6) as representative markers of the proximal tubules (Tsujimoto et al., 2020). The expression of glomerular and distal tubule markers was considerably lower in both 2D and 3D RPTEC spheroids than in human renal cortices, which contain both glomeruli and distal tubules. In contract, AQP1 expression in 2D RPTECs was as low as 26% of that in human renal cortices, whereas AQP1 and CDH6 were expressed at the same level in 3D RPTEC spheroids as in human renal cortices (Fig. 3A). This observation, based on the proteomics results, was further confirmed by the mRNA expression of additional markers (Supplemental Fig. 1), although there were some exceptions (such as CUBN for proximal tubules, LCN2 for distal tubules and CD2AP for podocytes).

OAT1 protein expression, used as the selection marker of culture condition, in 2D RTPECs and 3D RPTEC spheroids was 33% and approximately 65% of the average protein expression in the human renal cortices, respectively (Fig. 3B), and the protein expression was almost the same as that of mRNA. As is often the case with the down-regulation of proteins
occurring in cells with passages of several primary cells under conventional culture conditions, in 2D RPTECs, the expression of GLUT2, SGLT1, SGLT2, URAT1, OCTN1, OAT3, and OAT4 was less than 30% of the respective average protein expression in the human renal cortices (Fig. 3B). In the 3D RPTEC spheroids, out of the seven SLC transporters, proteins of GLUT2 and OCTN1 were increased to more than 30% of the average protein expression in the human renal cortices (Fig. 3B). The protein expression of the five selected ABC transporters in 3D RPTEC spheroids ranged from 70% to 306%, compared to the respective average protein expression in human renal cortices and 2D RPTECs, which ranged from 47% to 396% (Fig. 3D). No significant change in protein expression was observed in proteins other than PEPT1, even when the culture period of 3D RPTEC spheroids was extended from 12 days to 20 days (Fig. 3A-D). A heat map of the protein expression profiles of all quantified enzymes and SLC/ABC transporters is shown in Supplemental Fig. 2. In addition to protein expression in the TL, which represents total protein expression in cells, protein expression in the plasma membrane (PM) fraction, an indicator of functional transporters in the PM, was also investigated in 3D RPTEC spheroids (Fig. 4). The PM/TL ratios of cytosolic proteins were below 0.7, and those of Na⁺/K⁺-ATPase and P-gp, which were confirmed to be expressed mainly in the PM of the 3D RPTEC spheroids (Fig. 8A and B), were 7.54 and 7.09, respectively, indicating that the preparation of the PM fraction was carried out without any remarkable contamination. All ABC transporters and some SLC transporters such as PEPT1, GLUT9, OATP4C1, MATE1, OAT4, OCTN2, and OAT1 showed PM/TL ratios similar to those of Na⁺/K⁺-ATPase and P-gp, but far different from those of cytosolic proteins, suggesting that these transporters are mainly expressed in the PM. However, the PM/TL ratios for GLUT2, SGLT1, SGLT2, URAT1, OCTN1, OCT2, OAT3 and MATE2-K ranged from 1.28 to 4.00. SGLT1, SGLT2, URAT1 and OAT3 showed very low protein expression in both the TL (Fig. 3) and PM, indicating that these transporters were not abundantly expressed in
the 3D RPTEC spheroids. GLUT2 and OCT2 protein expression in the PM was low, although that in the TL was high, indicating that GLUT2 and OCT2 proteins are expressed not only in the PM but also in other parts of the 3D RPTEC spheroids.

**Effect of cisplatin and adefovir on intracellular ATP levels of 3D RPTECs**

Cisplatin and adefovir are substrates of OCT2/MATE1 and OAT1, respectively (Jongh et al., 2004; Izzedine et al., 2005). We investigated whether cisplatin and adefovir showed difference in toxicity between 2D RPTECs and 3D RPTEC spheroids and exhibited transporter-dependent toxicity in 3D RPTEC spheroids using intracellular ATP levels as an endpoint; because the dose-response to cefalotin and cisplatin on intracellular ATP levels showed a very similar response to those on lactate dehydrogenase release, which is often used to evaluate cell viability (Supplemental Fig. 3). Cisplatin showed a similar dose-response to intracellular ATP levels in 2D RPTECs and 3D RPTEC spheroids (Fig. 5), whereas the in vitro toxicity of adefovir was weaker in 2D RPTECs than in 3D RPTEC spheroids (Fig. 5). Cisplatin showed a concentration-dependent decrease of intracellular ATP levels and the addition of 1,000 µM cimetidine—an OCT and MATEs inhibitor—significantly suppressed the decrease in ATP compared to that in the absence of cimetidine, while 1,000 µM probenecid—an OAT inhibitor—did not cause a change (Fig. 6A). Adefovir reduced the intracellular ATP levels in a concentration-dependent manner. The decrease of intracellular ATP levels was not weakened by the addition of 1,000 µM cimetidine, while the addition of 1,000 µM probenecid almost completely diminished the adefovir-dependent decrease of intracellular ATP levels (Fig. 6B).

**Transport function in 3D RPTEC spheroids using metformin, furosemide, adefovir and digoxin as probe substrates**
The transporter function in the 3D RPTEC spheroids was evaluated using the following substrates: metformin for OCT2/MATEs, furosemide and adefovir for OAT1 and digoxin for P-gp (Uwai et al., 2007; Ebner et al., 2015) (Fig. 7). The uptake of metformin by 3D RPTEC spheroids was decreased by the OCT/MATEs inhibitor cimetidine, but only very weakly decreased by the OAT inhibitor probenecid. Probenecid did not significantly decrease the uptake of furosemide or adefovir, nor did it increase the uptake of digoxin. The P-gp inhibitor zosuquidar did not show a statistically significant increase in digoxin uptake; whereas cimetidine significantly increased digoxin uptake.

**P-gp and Na\(^+\)/K\(^+\) ATPase localization in 3D RPTEC spheroids**

P-gp and Na\(^+\)/K\(^+\)-ATPase are expressed on the apical and basolateral membrane sides of proximal renal tubular cells, respectively (Rostgaard and Møller, 1980; Launay-Vacher et al., 2006). The structure of 3D RPTEC spheroids was investigated using confocal laser scanning microscopy with the respective antibodies. One 3D RPTEC spheroid was approximately 200-300 µm in size, and the apical membrane marker P-gp was mainly detected on the outside of the 3D RPTEC spheroids and in the non-cell-to-cell contact area (Fig. 8A; green). P-gp was not highly expressed at the center of 3D RPTEC spheroids. In contrast, Na\(^+\)/K\(^+\)-ATPase, a marker of basolateral membranes, was mainly expressed inside 3D RPTEC spheroids and in the area where the cells were in contact with each other (Fig. 8B; red).

8. Discussion
Various in vitro tools have been developed for toxicity and pharmacokinetic assessments in the kidney (Faria et al., 2019). However, few reports remain on in vitro tools with sufficient transporter functions, such as OAT, even in 3D (Nieskens et al., 2016; King et al., 2017). In this study, we aimed to develop and evaluate a simple culture method with high throughput for RPTECs that express important transporters for pharmacokinetic and toxicity assessments.

When protein expression was measured as an endpoint, 90% of the detected 4,848 proteins in 3D RPTEC spheroids were within the 5-fold range of protein expression in the human renal cortices (Table 3). For SLC transporters, the percentage of transporters that were not within the 5-fold range of human renal cortices was approximately 30% in the 2D RPTECs, which was approximately 2-fold higher than that of enzyme and ABC transporters (Table 3). The 3D spheroid culture improved SLC transporter expressions by approximately 8%. However, the expression of transporters, such as URAT1/OAT4 (important transporters for the renal reabsorption of uric acid (Sato et al., 2008)), and SGLT1/SGLT2 (important transporters for glucose reabsorption), was as low as that of OAT3. OAT1, OAT3, OAT4, URAT1 and SGLT2 have been reported to be positively modified by hepatocyte nuclear factor 1 (HNF1) and negatively modified by DNA methylation (Kikuchi et al., 2006, 2007; Saji et al., 2008; Jin et al., 2012; Takesue et al., 2018). HNF1α/β protein expression was not detected by the LC-MS/MS proteomics analysis, but mRNA expression in 2D RPTECs and 3D RPTEC spheroids was similar to those in human renal cortices (data not shown). This suggests that the low expression of these transporters is not due to the low expression of HNF1 but because of DNA methylation. Saji et al., (2008) reported that OAT1 and OAT3 were regulated in clusters. The results of the present study differ from those of Saji et al. (2008) in that the expression of OAT1, but not OAT3, was upregulated by 3D spheroid culture of RPTECs. Therefore, it is reasonable to consider that methylation of the
post-promoter region, rather than of the promoter region, occurs in the 3D RPTEC spheroids. It is also possible that factors other than HNF1 and methylation are involved in the regulation of OAT1/3 expression. Further investigations are necessary to better understand the regulation of OAT1/3 expression in RPTECs.

Cisplatin decreases glomerular filtration rate and increases urinary albumin at clinical concentrations of approximately 10 µM unbound cisplatin (Erdlenbruch et al., 2001). Cisplatin showed a concentration-dependent decrease in intracellular ATP levels in 3D RPTEC spheroids, and a 50% reduction of intracellular ATP was obtained at 10 µM which is the clinical plasma-free cisplatin concentration (Fig. 6A). Intracellular ATP levels in HK-2 cells, which do not express OCT2 or MATE (Sancho-Martínez et al., 2011; Hauwaert et al., 2014), were reduced to about half at 30-100 µM cisplatin. To understand why almost the same concentration decreased intracellular ATP levels by half in HK-2 and 3D RPTEC spheroids although the expression of OCT/MATE transporters was different, two points should be taken into consideration. First, the contribution of passive diffusion of basic drugs is larger than that of acidic drugs because passive diffusion is affected by the internal negative membrane potential. Secondly, longer exposure times are generally used in in vitro toxicity studies than in in vitro transporter studies. The intracellular concentration of cisplatin is unlikely to differ greatly between 3D RPTEC spheroids and HK-2 cells, because the impact of the OCT2/MATE process is not large in in vitro toxicity studies.

Adefovir (120 mg/day) has been reported to cause nephrotoxicity in 20-30% of patients when administered at a clinic for 48 weeks (Kahn et al., 1999; Fisher et al., 2001). The intracellular ATP level in 3D RPTEC spheroids was reduced by adefovir in a concentration-dependent manner, with the amount of intracellular ATP reduced to about half of the control at concentrations of 100-1,000 µM (Fig. 6B). The addition of the OAT inhibitor probenecid completely suppressed the decrease in cellular ATP, suggesting that adefovir
toxicity observed in 3D RPTEC spheroids is a function of the OAT transporter. The in vitro cytotoxicity of adefovir was also evaluated with RPTECs in which OAT1 was transfected using cell viability as an endpoint and the obtained IC50 value was 230 µM (Nieskens et al., 2016). The clinical unbound Cmax of adefovir was approximately 1 µM after 125 mg/day (Barditch-Crovo et al., 1997) and nephrotoxicity was seen in 20-30% of patients. The discrepancy may be explained by exposure period with adefovir is not sufficient because the clinical toxicity of adefovir has been observed 48-72 weeks post-dose in clinics, and the in vitro toxicity of cisplatin is reported to be enhanced 100-fold by increasing the duration of drug exposure from 24 h to 14 days (Secker et al., 2019). This study revealed that 3D RPTEC spheroids maintained their morphology over 5-20 days and the gene expression did not significantly change from 12 to 32 days (Fig. 2), thus the toxic effects of long-term exposure can also be examined in 3D RPTEC spheroids.

In addition to the OCT/MATE-dependent in vitro cytotoxicity of cisplatin in 3D RPTEC spheroids (Fig. 6), cimetidine reduced the uptake of metformin into 3D RPTEC (Fig. 7). This was consistent with the proteomics results showing that the expression levels of OCT2, MATE1, and MATE-2K in 3D RPTEC spheroids were within the range of variation in their expression levels in human renal cortices, and their expression was confirmed in the PM fraction (Figs. 3B and 4A). In contrast, no uptake of adefovir or furosemide by OAT1 was observed, although OAT1 protein expression was within the range of variation in protein expression in the human renal cortices, was observed in PM fractions and the in vitro cytotoxicity of adefovir in 3D RPTEC spheroids was OAT-dependent. Immunostaining revealed that P-gp, a marker of apical membranes, was strongly expressed on the outer side of the 3D RPTEC spheroids and in areas where the RPTECs were not in contact with each other. However, P-gp expression decreased toward the center of the 3D RPTEC spheroids. Na+/K+-ATPase, a basolateral membrane marker, was strongly expressed in areas where RPTECs...
were in contact with each other, and its expression in the center of the 3D RPTEC spheroids was not as weak as that of P-gp (Fig. 8). The following two possibilities were considered as reasons why basolateral OAT1 activity was not detected in 3D RPTEC spheroids: 1) apical and basal transporter activities cannot be evaluated separately because both apical transporters are exposed to the buffer as well as in vitro kidney slice systems (Arakawa et al., 2017, 2019), 2) a wash process in the in vitro transporter assay is not sufficient for transporters on the basolateral side because transporters on the basolateral side of RPTECs are expressed in the area where cells are in contact. Genetic polymorphisms can weaken the correlation between activity and protein expression. However, for OAT1, although SNP with a complete loss of function (R454Q) has been reported, the allele frequency is extremely low (0.2%) (Fujita et al., 2004). Therefore, it is unlikely that the SNPs in OAT1 caused a discrepancy between OAT1 protein expression and the undetected OAT activity in the 3D RPTEC spheroids, although unidentified SNPs may be involved. Further efforts are necessary to precisely evaluate transporter activity in the future.

Reproducibility is an important factor in evaluating the usefulness of an in vitro system. The reproducibility of the 3D RPTEC spheroids was evaluated by comparing the CV% of mRNA expression in 3D RPTEC spheroids from three different lots of RPTECs at a culture period of 12-14 days. Furthermore these CV% values were compared with those of human kidney cortex from five different donors (Supplemental Table 4). Of the 16,993 genes detected, 87% showed variation within 50 (CV%), which was also true for 3,193 enzymes, 334 SLC transporters, and 39 ABC transporters in the case of 3D RPTEC spheroids, whereas CV% for all genes, enzymes, SLC transporters, and ABC transporters in the human kidney cortex were 75%, 80%, 61%, and 80%, respectively. This indicates that the 3D culture method used in this study is a highly reproducible culture condition with a small variation in mRNA expression among the three different lots of RPTECs (from three different donors).
compared with the variation in mRNA expression in the human kidney cortex from five different donors.

In summary, we have confirmed that a spheroidal culture condition, selected by monitoring OAT1 gene expression, improved the protein expression of other genes to be more similar to that in human kidney cortices, resulting in a similar protein expression level of 90% of more than 4,800 proteins detected over an extended period of up to 20 days in culture. This condition also enabled the evaluation of not only transporter-dependent cisplatin nephrotoxicity, which has been evaluated in 2D culture conditions and other cell systems, but also transporter-dependent adefovir nephrotoxicity more sensitively. In vitro 3D RPTEC spheroids could positively impact preclinical drug discovery/drug development, helping to prevent unwanted failures in late-stage drug development including clinical trials. However, the polarized expression of transporters has not yet been fully evaluated in the current system and genetic polymorphisms may interfere the comparison of mRNA and protein expression between RPTECs and human kidney cortices. Moreover, the current system is a static culture, and the inclusion of laminar flow on the RPTEC could further enhance tubular epithelial cell function.

9. Acknowledgments

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10. Authorship contributions

Participated in research design: E. Takahashi, Kitagawa, Kondo, Jimbo, Saito, Ishiguro, Takatani, R. Takahashi, Morinaga, Kudo, Arakawa, Tamai, Mae, and Osafune

Conducted experiments: E. Takahashi, Kitagawa, Kadoguchi, Higuchi, Nakazone, Saito, Takatani, R. Takahashi, Kudo and Morinaga

Performed data analysis: Kadoguchi, Higuchi, Nakazone, E. Takahashi, Kitagawa, Saito, Takatani, R. Takahashi, Kudo, and Morinaga

Wrote or contributed to the writing of the manuscript: E. Takahashi, Kondo, Saito, Morinaga, Ishiguro, Osafune, and Tamai

11. References


jPOSTrepo: an international standard data repository for proteomes Shujiro Okuda; Yu Watanabe; Yuki Moriya; Shin Kawano; Tadashi Yamamoto; Masaki Matsumoto; Tomoyo Takami; Daiki Kobayashi; Norie Araki; Akiyasu C. Yoshizawa; Tsuyoshi Tabata; Naoyuki Sugiyama; Susumu Goto; Yasushi Ishihama Nucleic Acids Research 45 (D1): D1107-D1111 (2017). doi: 10.1093/nar/gkw1080


Recapitulate Key Aspects of Renal Physiology to Enable Nephrotoxicity Testing. *Front Physiol* 8:123.


12. Footnote

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13. Figure Legends

Figure 1  OAT1 mRNA expression in 3D RPTEC spheroids at various culture days (A) and various seeding densities (B)

(A) RPTEC (lot: 664995) were cultured in spheroidal aggregate for 0, 2, 4, 6, 8, and 13 days at a cell number of 1,000 cells/spheroid. (B) RPTECs (lot: 664995) were seeded at a cell number of 500, 1,000, 2,500, 5,000, 10,000, 25,000, and 50,000 cells/spheroid and cultured for 12 days. mRNA expression of OAT1 was determined on day 12. Bar represents the average ± SD of three replications on three different days.

Figure 2  mRNA expression of drug transporters in 2D RPTECs (A), 3D RPTEC spheroids (B), and human cortical tissues (C) and comparison of mRNA expression profiles of enzymes (D), SLC transporters (E), and ABC transporters (F) in 2D RPTECs and 3D RPTEC spheroids with those in human cortical tissues.

(A) 2D RPTECs (lot: 664995) were cultured for 2 and 12 days. (B) 3D RPTEC (lot: 664995) was cultured in spheroidal aggregates for 5, 12, 20, and 32 days. (C) mRNA expression of typical drug transporters in five donors of human kidney cortical tissues. Bar represents the mean ± SD of five different donors. (D-F) the number represents protein (%) to the total number of genes. 2D, 2D RPTECs; 3D, 3D RPTEC spheroids.

Figure 3  Protein expression of kidney markers (A), SLC transporters (B and C), and ABC transporters (D).

Protein abundance was expressed as % of the human kidney cortex 1. The protein abundance of human kidney cortices represents the mean ± SD of five different donors. Human cortex, human kidney cortex.
Figure 4  Protein expression ratio of SLC transporters (A), ABC transporters (B), and cytosolic proteins in PM and TL fractions of 3D RPTEC spheroids.

The PM-to-TL ratio was calculated from the protein abundance (% of human kidney cortex 1 in the TL) of each protein in the PM and TL. RPTECs (lot. 664995) were cultured in 3D spheroid culture for 12-14 days. The PM/TL ratio of Na+/K+-ATPase was 7.54. CBR1, carbonyl reductase 1; ALDH2, aldehyde dehydrogenase 2; SHMT1, serine hydroxymethyltransferase 1; ACAT2, acetyl-CoA acetyltransferase 2; G6PD, glucose 6 phosphate dehydrogenase. The PM/TL ratios of proteins detected are shown in supplemental table 5.

Figure 5  Effect of cisplatin (A) and adefovir (B) on cellular ATP contents in 2D RPTECs and 3D RPTEC spheroids.

2D RPTECs and 3D RPTEC spheroids (lot: 664995) were exposed to cisplatin (0, 1, 3, 10, 30 and 100 µM) or adefovir (0, 1, 3, 10, 30, and 100 µM) for 7 days. Each bar represents the mean ± SD of four replications. * Statistically significant compared to the control, $p<0.05$, Dunnett’s test.

Figure 6  Effect of cisplatin (A) and adefovir (B) on cellular ATP contents in the absence and presence of the OCT inhibitor cimetidine and the OAT inhibitor probenecid.

3D-RPTECs (lot: 664995) were exposed to cisplatin (0, 3, 10, and 30 µM) or adefovir (0, 10, 100, and 1,000 µM) for 3 days in the absence or presence of cimetidine (1,000 µM) or probenecid (1,000 µM). Each bar represents mean ± SD of four replications. * Statistically significant compared to the control, $p<0.05$, Dunnett’s test.
Figure 7  Uptake of metformin (A), furosemide (B), adefovir (C), and digoxin (D) in 3D RPTEC spheroids in the presence and absence of typical inhibitors.

Inhibitor concentrations of probenecid, cimetidine and zosuquidar were 100/1,000 μM (A, B and D/C), 4,000 μM, and 1 μM, respectively. * Statistically significant, t-test, RPTECs (lot: 664995) were used.

Figure 8  Representative immunofluorescence images of P-gp (green; A), Na⁺-K⁺ ATPase (red; B), and nuclei (blue; C) in 3D RPTEC spheroids.

P-gp, Na⁺/K⁺-ATPase and nuclei were stained with rabbit polyclonal anti-P-gp antibody (visualized using anti-rabbit Alexa Fluor 488), goat polyclonal anti-Na⁺/K⁺-ATPase antibody (visualized using anti-goat Alexa Fluor 647) and DAPI, respectively. Images were captured at the original magnification (x20). Scale bars, 20 μm. RPTECs (lot: 664995) were used.
Table 1  Donor information of human kidneys and RPTECs

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<th>Ethnic background</th>
<th>Type</th>
<th>PMI, hours</th>
<th>BMI</th>
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<th>Comorbidities</th>
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<td>64</td>
<td>Caucasian</td>
<td>Biopsy</td>
<td>-</td>
<td>-</td>
<td>-</td>
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Post-mortem interval (PMI): the time elapsed since an individual's death.

CAD: coronary artery disease

Table 2  Equivalence between 3D RPTEC spheroids (N=3) and human kidney cortices (N=5) based on mRNA expression

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<td>No. of genes*</td>
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*: Values in the table represent number of genes within 90% confidence intervals of the mRNA expression ratio between 3D RPTEC spheroids (day 12 or 14, from different three donors) and human kidney cortices (N=5).
Table 3 Difference of the protein expression profiles of 2D RPTECs and 3D RPTEC spheroids (cultured for 12 days) compared to human cortical tissues (N=5)

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<td>6.5</td>
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Values in the table show % of total proteins in the respective fold change.

15. Figures

Separate files
Supplemental Data

Separate files
Figure 1

(A) OAT1 mRNA fold change from day 0 vs. Culture period (day).

(B) OAT1 mRNA fold change from 1000 cells vs. Cell number.
Figure. 3

(A) Protein abundance (% of cortex 1)

(B) Protein abundance (% of cortex 1)

(C) Protein abundance (% of cortex 1)

Podocyte Proximal tubular cells Distal tubular cells

RPTEC 2D Day 12
RPTEC 3D Day 12
RPTEC 3D Day 20
Human cortex

(D) Protein abundance (% of cortex 1)

ABC1
ABC3
ABC5
ABC9

Figure. 5

Intracellular ATP (% of control) vs. Cisplatin concentration (µM)

- 3D RPTEC spheroids
- 2D RPTEC

Intracellular ATP (% of control) vs. Adefovir concentration (µM)

- 3D RPTEC spheroids
- 2D RPTEC
Figure 6

(A) and (B) show the intracellular ATP levels in cells treated with Cisplatin or Adefovir, respectively, with or without Cimetidine or Probenecid. The bars indicate the mean ATP levels, and the error bars represent the standard deviation. Asterisks denote statistically significant differences compared to the control group.

(A) Cisplatin (µM)
- Control
- +Cimetidine
- +Probenecid

(B) Adefovir (µM)
- Control
- +Cimetidine
- +Probenecid

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**Figure. 7**

### Table

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<thead>
<tr>
<th>Substance</th>
<th>Control</th>
<th>Probenecid</th>
<th>Cimetidine</th>
<th>Zonisamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin uptake (µL/10 min/mg)</td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
<tr>
<td>Furosemide uptake (µL/min/mg)</td>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
<td><img src="image7.png" alt="Graph" /></td>
<td><img src="image8.png" alt="Graph" /></td>
</tr>
<tr>
<td>Adefovir uptake (µL/2 min/mg)</td>
<td><img src="image9.png" alt="Graph" /></td>
<td><img src="image10.png" alt="Graph" /></td>
<td><img src="image11.png" alt="Graph" /></td>
<td><img src="image12.png" alt="Graph" /></td>
</tr>
<tr>
<td>Digoxin uptake (µL/10 min/mg)</td>
<td><img src="image13.png" alt="Graph" /></td>
<td><img src="image14.png" alt="Graph" /></td>
<td><img src="image15.png" alt="Graph" /></td>
<td><img src="image16.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

* indicates a significant difference.
Figure 8