Journal of Pharmaceutical Sciences 105 (2016) 1011–1016

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Note

# Quantitative Targeted Absolute Proteomics for 28 Transporters in Brush-Border and Basolateral Membrane Fractions of Rat Kidney

Yasuo Uchida [1], Takafumi Toyohara [2], Sumio Ohtsuki [3], Yoshinori Moriyama [4, 5], Takaaki Abe [2, 6, 7], Tetsuya Terasaki [1, *]

[1] Division of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan
[2] Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
[3] Department of Pharmaceutical Microbiology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
[4] Department of Membrane Biochemistry, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan
[5] Advanced Science Research Center, Okayama University, Okayama, Japan
[6] Department of Clinical Biology and Hormonal Regulation, Tohoku University Graduate School of Medicine, Sendai, Japan
[7] Division of Medical Science, Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan

ARTICLE INFO

*Article history:*
Received 22 July 2015
Revised 24 August 2015
Accepted 26 August 2015
Available online 14 September 2015

*Keywords:*
proteomics
proteins
ABC transporters
solute transporters
membrane transport
membrane transporter
renal excretion
drug transport
HPLC (high-performance/pressure liquid chromatography)
mass spectrometry

ABSTRACT

The purpose of the present study was to determine the absolute protein expression levels of various transporters in renal brush-border membrane (BBM) and basolateral membrane (BLM) fractions, in order to understand the quantitative differences in average transport activities among different transporters at each cellular membrane. BBM and BLM fractions of rat kidney were prepared and digested with trypsin, and simultaneous absolute quantification of 28 transporters and a BLM marker, $Na^+/K^+$-ATPase, was performed using our established quantitative-targeted absolute proteomics (QTAP) technique. In BBM fraction, the protein expression levels of bcrp, urat1, mate1, octl1, mrp4, mdr1a, and abca3 were 40.3, 22.2, 8.90, 4.85, 4.69, 3.22, and 0.976 fmol/μg protein, respectively. In BLM fraction, the protein expression levels of oat1, oat3, oct1, mrp6, and mrp1 were 10.6, 10.2, 4.59, 0.724, and 0.271 fmol/μg protein, respectively. The expression levels of abca2, abca4, abca5, abca12, abcb4, mrp5, abcc9, abcg1, abcg5, lat1, ntcp, pgt, oatp2b1, oatp1b2, oatp3a1, and oct3 were under the limit of quantification in both fractions. The quantitative transporter protein expression profiles at these membranes, as determined by QTAP analysis, should be helpful to understand the contributions of individual transporters to renal excretion of xenobiotics and endogenous compounds.



## Introduction

Renal tubular epithelial cells express a variety of transporters and regulate the tubular secretion and reabsorption of xenobiotics and endogenous compounds. The qualitative expression profiles of transporters are greatly different at the brush-border membrane (BBM) and basolateral membrane (BLM) of the epithelial cells, so that most compounds are likely to cross each membrane via different transporters in the secretion and reabsorption processes.[1] *In vivo* studies, especially ones using gene-knockout mice, have revealed the roles and contributions of individual transporters to secretion and reabsorption.[2-7] However, there has been no comprehensive investigation about the quantitative transport activities of the many different transporters at each cellular membrane, though this information would be helpful to predict renal clearance of xenobiotics and endogenous compounds, as well as drug-drug interactions.

The transport activities of transporters localized at the BLM have been well evaluated by means of integration-plot analysis, the

*Abbreviations used:* abc, ATP-binding cassette; BBB, blood-brain barrier; BBM, brush-border membrane; BLM, basolateral membrane; CM, crude membrane; LC-MS/MS, liquid chromatography-tandem mass spectrometry; QTAP, quantitative targeted absolute proteomics; slc, solute carrier; SRM/MRM, selected/multiple reaction monitoring.

This article contains supplementary material available from the authors upon request or via the Internet at http://dx.doi.org/10.1002/jps.24645.

* *Correspondence to:* Tetsuya Terasaki (Telephone: +81-22-795-6831; Fax: +8122-795-6886).
*E-mail address:* terasaki.tetsuya@m.tohoku.ac.jp (T. Terasaki).

http://dx.doi.org/10.1002/jps.24645
0022-3549/

kidney uptake index method, kidney slice uptake study, and other methods.[6,8-14] Therefore, it is roughly understood which transporters have the greatest impact on drug transport across the BLM. It is considered that organic anion transporter (oat) 1 and oat3 mainly contribute to the uptake of anionic drugs by epithelial cells from blood.[15] The BBM also expresses many transporters, including multidrug resistance protein (mdr) 1a/P-glycoprotein (P-gp), breast cancer resistance protein (bcrp), and multidrug resistance-associated protein (mrp) 4, which have been shown to contribute to drug transport by means of studies in gene-knockout mice.[2,4,16] However, the differences in average transport activities among different transporters are poorly understood, in contrast to BLM transporters.

In 2008, we established a quantitative targeted absolute proteomics (QTAP) technique able to simultaneously quantify the protein expression levels of 37 transporters, and used it to clarify the differences in absolute protein expression levels among transporters at the mouse blood-brain barrier (BBB).[17] Efflux of hydrophobic drugs into blood is often mediated by both mdr1a and bcrp at the BBB. We found that the protein expression level of mdr1a was 3.2-fold greater than that of bcrp at mouse BBB,[17] suggesting a greater role of mdr1a than bcrp in efflux. Indeed, the increases in brain-to-plasma concentration ratios of common substrates are greater in the case of mdr1a gene-knockout mice than bcrp gene-knockout mice.[18] Therefore, the QTAP technique is expected to help us to understand the differences in transport activities among transporters.

We have already reported the protein expression levels of transporters in whole plasma membrane fraction of mouse kidney.[17] However, because this fraction includes BLM and intracellular membrane, the data might not adequately reflect the *in vivo* quantitative expression profile at the BBM. The situation is similar for the BLM. Oat3 is considered to contribute to the uptake of various anionic drugs to a similar degree to oat1,[15] but the reported expression level of oat3 in the whole plasma membrane fraction is about threefold lower than that of oat1,[17] emphasizing the need to conduct QTAP analysis using purified BLM fraction.

Therefore, the purpose of the present study was to determine the protein expression levels of multiple transporters in purified BBM and BLM fractions of kidney.

## Materials and Methods

### *Rats*

Male Wistar rats were maintained on a 12-h light/dark cycle in a temperature-controlled environment with free access to food and water, and used for experiments at 6-7 weeks of age. These rats were the same ones as the nontransgenic littermates employed as a negative control in the human SLCO4C1 study reported by Toyohara et al.[19] It was confirmed that they were genetically normal. The reason why these nontransgenic littermate rats were used is because the present study was conducted as one of a series of projects related to the study reported by Toyohara et al.[19] All animal experiments were approved by the Tohoku University Animal Care Committee, and were performed in accordance with the guidelines of Tohoku University.

### *Preparation of Crude Membrane, BBM, and BLM Fractions of Rat Kidney*

Crude membrane (CM),[20] BBM,[21] and BLM[22] fractions were prepared as described previously with some modifications. Briefly, for CM fraction, the excised rat kidneys were homogenized in a buffer containing 0.23 M sucrose, 5 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A. The homogenate was centrifuged at 3000*g* for 15 min, and the supernatant was further centrifuged at 100,000*g* for 30 min (the pellet is CM fraction). For BBM fraction, the kidneys were homogenized in a buffer containing 10 mM Tris-HEPES (pH 7.4), 10 mM $CaCl_2$, 30 mM mannitol, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A. The homogenate was allowed to stand on ice for 15 min, and then centrifuged at 3000*g* for 10 min. The supernatant was centrifuged at 113,613*g* for 45 min with a Beckman Type 60Ti rotor (the pellet is the BBM fraction). For BLM fraction, the kidneys were homogenized in a buffer A (0.3 M sucrose, 10 mM MOPS-NaOH (pH 7.5), 5 mM EDTA-NaOH, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A), and then centrifuged at 3000*g* for 10 min. The supernatant was centrifuged at 20,500*g* for 15 min with an RP46 rotor in Hitachi Himac CR20G. The pellet was resuspended in buffer A, and Percoll was added to a concentration of about 10% (v/v), then the suspension was centrifuged at 54,455*g* for 30 min with a Beckman SW4lTi rotor. The upper half of the supernatant was centrifuged at 102,536*g* for 60 min with a Beckman Type 60Ti rotor. The pellet was resuspended in buffer A, and centrifuged at 102,536*g* for 60 min again (the pellet is the BLM fraction).

### *Liquid Chromatography-Tandem Mass Spectrometry With Multiplexed Selected/Multiple Reaction Monitoring Analysis*

Absolute quantitation of 28 transporters and $Na^+/K^+$-ATPase was simultaneously performed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using our previously described multiplexed selected/multiple reaction monitoring (SRM/MRM) analysis.[23,24] The experimental and analytical procedures were the same as those described in Uchida et al.[23] Briefly, the membrane fractions were trypsinized under denaturing conditions, spiked with stable isotope-labeled peptides, and injected into the LC-MS/MS system, which is an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) coupled with a triple-quadrupole mass spectrometer (API5000; AB SCIEX, Foster City, CA) equipped with a Turbo V ion source (AB SCIEX). An Agilent 300SB-C18 (0.5 mm × 150 mm, 5.0 μm) was used as an analytical column. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The peptides were separated and eluted from the column at room temperature using a linear gradient with a 120-min run time at a flow rate of 50 μL/min. The sequence was as follows: (A : B), 99 : 1 for 5 min after injection, 50 : 50 at 55 min, 0 : 100 at 56 min and up to 58 min, 99 : 1 at 60 min and up to 120 min. The targeted peptides eluted from the HPLC column were simultaneously and selectively detected by means of electrospray ionization in a multiplexed SRM/MRM mode. Each molecule was monitored with four sets of SRM/MRM transitions (Q1/Q3-1, Q1/Q3-2, Q1/Q3-3, Q1/Q3-4) derived from one set of native (standard) and stable isotope-labeled (internal standard) peptides. Five transporters [ATP-binding cassette (abc) b4, abcc9, solute carrier (slc) 21a10, slc21a9, and slc22a13] were monitored using the peptides and SRM/MRM transitions listed in Supplemental Table 1, and the other molecules were monitored using those reported by Uchida et al.[25] Signal peaks with a peak area count of over 5000 detected at the same retention time as an internal standard peptide were defined as positive. When three or four out of four sets of SRM/MRM transitions showed positive peaks, the molecule was considered to be expressed in the sample, and the protein expression level was determined as the average of the three or four quantitative values. The LC-MS/MS analysis was performed four times, and the protein expression levels of target molecules were finally calculated as the average values of the four samples. The limit of quantification was calculated as described previously.[23] Kamiie et al.[17] have previously validated that protein expression levels exhibit coefficients of variation of less than 20.0% when determined from