Human drug transporter protein expression in lung: Impact of quantitative targeted absolute proteomics (QTAP) for drug development in pulmonary disease

Atsushi Sakamoto\textsuperscript{1,2}, Norio Yamamura\textsuperscript{1} and Tetsuya Terasaki\textsuperscript{2}

\textsuperscript{1} Pharmacokinetics and Nonclinical Safety, Nippon Boehringer Ingelheim, Kobe, JAPAN
\textsuperscript{2} Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, JAPAN
Conflict of Interests
Tetsuya Terasaki is a Director of Proteomedix Frontiers Co., Ltd. This study was not supported by Proteomedix Frontiers Co., Ltd., and his positions at Proteomedix Frontiers Co., Ltd. did not influence the design of the study, the collection of the data, the analysis or interpretation of the data, the decision to submit the manuscript for publication, or the writing of the manuscript and did not present any financial conflicts.

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Approval by the Ethical Committee
The research protocol in the present studies were approved by the ethical committees of Nippon Boehringer Ingelheim Co. Ltd. (NBI) and Graduate School of Pharmaceutical Sciences, Tohoku University.
Quantitative Atlas of Membrane Transporter Proteins: Development and Application of a Highly Sensitive Simultaneous LC/MS/MS Method Combined with Novel In-silico Peptide Selection Criteria

Junichi Kamiie,1,2 Sumio Ohtsuki,1,2 Ryo Iwase,1 Ken Ohmine,1 Yuki Katsukura,1,2 Kazunari Yanai,1 Yumi Sekine,1 Yasuo Uchida,1 Shingo Ito,1,2 and Tetsuya Terasaki1,2,3

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Purpose. To develop an absolute quantification method for membrane proteins, and to construct a quantitative atlas of membrane transporter proteins in the blood–brain barrier, liver and kidney of mouse.

Methods. Mouse tissues were digested with trypsin, and mixed with stable isotope labeled-peptide as a quantitative standard. The amounts of transporter proteins were simultaneously determined by liquid chromatography–tandem mass spectrometer (LC/MS/MS).

Results. The target proteins were digested in-silico, and target peptides for analysis were chosen on the basis of the selection criteria. All of the peptides selected exhibited a detection limit of 10 fmol and linearity over at least two orders of magnitude in the calibration curve for LC/MS/MS analysis. The method was applied to obtain the expression levels of 34 transporters in liver, kidney and blood–brain barrier of mouse. The quantitative values of transporter proteins showed an excellent correlation with the values obtained with existing methods using antibodies or binding molecules.

Conclusion. A sensitive and simultaneous quantification method was developed for membrane proteins. By using this method, we constructed a quantitative atlas of membrane transporter proteins at the blood–brain barrier, liver and kidney in mouse. This technology is expected to have major implications for various fields of biomedical science.

KEY WORDS: ABC transporter; LC/MS/MS; multiple reaction monitoring (MRM); pharmacoproteomics; SLC transporter.
Principle of the Multiplexed-MRM and Shot-Gun LC-MS/MS method for the protein quantification

1. Calibration
   - Native peptide
   - Heavy peptide (C13, N15)
   - Internal standard peptide
   - Selection of specific peptide fragment

2. Sample pre-treatment
   - Denaturation of Protein
   - Reduction + Alkilation
   - Trypsin digestion

3. Sample analysis
   - Addition of heavy peptide
   - LC-MS/MS MRM analysis
   - Quantification
Multiple/Selective Reaction Monitoring mode enable us to cut off significant noise peak coming from proteins which does not need to detect. Now, we could detect 1 fmol → 10 amol transporter protein per assay including phospholylated or modified protein.

Example of multiplexed-MRM analysis

**St peptide channel**

- Channel 1: 460.3/796.3, 35.2 min, 233 ± 9 fmol/µg protein
- Channel 2: 460.3/685.3, 35.2 min, 224 ± 19 fmol/µg protein
- Channel 3: 460.3/571.7, 35.2 min, 245 ± 20 fmol/µg protein
- Channel 4: 460.3/500.8, 35.2 min, 226 ± 14 fmol/µg protein

**IS peptide channel**

- Channel 1: 462.3/800.3, 35.2 min
- Channel 2: 462.3/689.3, 35.2 min
- Channel 3: 462.3/575.7, 35.2 min
- Channel 4: 462.3/504.8, 35.2 min

**Average:** 232 fmol/µg protein
A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood–brain barrier in ddY, FVB, and C57BL/6J mice

Yasuo Uchida¹, Masanori Tachikawa¹, Wataru Obuchi¹, Yutaro Hoshi¹, Yusuke Tomioka¹, Sumio Ohtsuki² and Tetsuya Terasaki¹*
Ultimate goal of pharmacoproteomics (PPx) is to understand organ function both in normal and diseased human by knowing the absolute protein expression amount of enzyme, transporter, channel and receptors in the cells and their intrinsic activities.
Lung is very important organ both for the target organ of pulmonary disease and the absorption organ of systemic administration. Therefore, important subjects to be studied are …

Lung consists of different cells

Questions

1. Name of transporters ?
2. Amount of transporter protein ?
3. Differences of regional, interindividual and gender ?
4. Transporter protein amount correlate with its activity ?
5. Best immortalized cell line for the in vitro study ?
Purpose

1. To clarify the expression levels of transporter proteins in human lung tissue and to analyze regional, interindividual and gender differences in primary cultured epithelial cells.

2. To evaluate whether differences in OCTN1 and MRP1 protein expression govern the respective transport activity in primary cultured human lung cells.

3. To clarify the drug transporter protein expression of five commercially available immortalized lung cell lines derived from tracheobronchial cells (Calu-3 and BEAS2-B), bronchiolar-alveolar cells (NCI-H292 and NCI-H441), and alveolar type II cells (A549), by liquid chromatography-tandem mass spectrometry-based approaches.
Frozen whole lung tissues were purchased from Analytical Biological Services. Normal human bronchial epithelial cells (NHBEs) were purchased from Lonza. Human tracheal epithelial cells (HTEpiCs) and human pulmonary alveolar epithelial cells (HPAEPiCs) were purchased from ScienCell.
List of transporter proteins quantified

Those transporters that mRNA expression was reported (Bosquillon C., J Pharm Sci. 2010) were selected for the protein quantification by LC-MS/MS.

**ABC transporters**
- MDR1, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, MRP7
- MRP8, MRP9, BCRP

**SLC transporters**
- OCT1, OCT2, OCT3, OCTN1, OCTN2, OAT1, OAT2, OAT3, OAT4
- PEPT1, PEPT2

**SLCO transporters**
- OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1, OATP4A1, OATP1C1, OATP5A1, OATP6A1, OATP4C1, PGT (OATP2A1)
MRP1 express abundantly in bronchi and alveoli

It is reported that MRP1 is localized in the basolateral membrane of lung epithelial cells (Scheffer et al. J Clin Pathol 2002, Bosquillon C., J Pharm Sci. 2010), suggesting that MRP1 may play an important role in the systemic availability of inhaled drug.

Expression levels of transporter proteins were determined in purified plasma membrane fractions.
Data represent mean ± SD (7 donors: tissues, 5 donors: cells).

OCTN1 express abundantly in all lung region.

It is reported that OCTN1 is localized in the apical membrane of lung epithelial cells (Horvath et al. Am J Repir Cell Mol Biol 2007), suggesting that OCTN1 may play an important role in the uptake of inhaled drug into the epithelial cells.

Expression levels of transporter proteins were determined in purified plasma membrane fractions. Data represent mean ± SD (7 donors: tissues, 5 donors: cells).

OATP2B1 express in all lung region

OATP2B1 could be an useful target transporter of inhaled drug

Expression levels of transporter proteins were determined in purified plasma membrane fractions. Data represent mean ± SD (7 donors: tissues, 5 donors: cells).

Comparison of transporter protein expression among trachea, bronchi and alveoli

Expression levels of transporter proteins were determined in purified plasma membrane fractions. Data represent mean ± SD (5 donors). *p < 0.05, N.S. : not significant difference

Interindividual difference of transporter protein expression in trachea, bronchi and alveoli

Expression levels of transporter proteins were determined in purified plasma membrane fractions. Data represent mean ± SEM.

Expression levels of transporter proteins were determined in purified plasma membrane fractions. Data represent mean ± SD (3 donors: male, 4 donors: female).

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3. To clarify the drug transporter protein expression of five commercially available immortalized lung cell lines derived from tracheobronchial cells (Calu-3 and BEAS2-B), bronchiolar-alveolar cells (NCI-H292 and NCI-H441), and alveolar type II cells (A549), by liquid chromatography-tandem mass spectrometry-based approaches.
OCTN substrate, ASP\textsuperscript{+} was taken up by lung cells in a concentration-dependent manner, suggesting OCTN1 is acting significantly for the pulmonary drug uptake.

ASP\textsuperscript{+} (4-[4-(dimethyl-amino)styryl]-N-methylpyridinium iodide)
Individual differences in OCTN1 transport activity were highly correlated with protein expression levels in the plasma membrane fraction, suggesting OCTN1 protein concentrations are determinants for drug distribution in the lung.
Individual differences in MRP1 transport activity were highly correlated with protein expression levels in the plasma membrane fraction, suggesting MRP1 protein concentrations are determinants for drug distribution in the lung.

Primary cultured lung cells are considered to be the best experimental model for in vivo studies and to test new drugs, while they are more difficult to obtain and have limited growth activity.

Therefore, there is an increasing interest in immortalized lung cell lines as an alternative to primary cultured lung cells.

Calu-3 and BEAS2-B cell lines have been commonly used for the studies of metabolism and the interaction of cells with xenobiotics as models of tracheobronchial epithelial cells.

NCIH292 and NCI-H441 are derived from the human lung carcinoma and have characteristics similar to those of alveolar and bronchiolar epithelial cells.

A549 cells are the most commonly used in alveolar epithelial models.
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3. To clarify the drug transporter protein expression of five commercially available immortalized lung cell lines derived from tracheobronchial cells (Calu-3 and BEAS2-B), bronchiolar-alveolar cells (NCI-H292 and NCI-H441), and alveolar type II cells (A549), by liquid chromatography-tandem mass spectrometry-based approaches.
There were some inconsistencies found in protein expressions compared with mRNA expression reported previously.

Table 2. Comparison of Protein with mRNA Expression Profiles of Drug Transporters in Lung Cell Models

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Protein Expression</th>
<th>mRNA by Endter et al.(^{11}) (Day 8)</th>
<th>mRNA by Endter et al.(^{11}) (Day 15)</th>
<th>mRNA by Courcot et al.(^{12})</th>
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<tbody>
<tr>
<td>ABC Transporter</td>
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<tr>
<td>MDR1 (P-gp)</td>
<td>–</td>
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<tr>
<td>MRP1</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>MRP2</td>
<td>++</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>MRP3</td>
<td>–</td>
<td>++</td>
<td>+++</td>
<td>–</td>
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<tr>
<td>MRP4</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>MRP5</td>
<td>–</td>
<td>++</td>
<td>–</td>
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<tr>
<td>MRP6</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>MRP7</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
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<tr>
<td>BCRP</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>SLC Transporter</td>
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<td></td>
</tr>
<tr>
<td>OCT1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>OCT2</td>
<td>–</td>
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<td>OCT3</td>
<td>+</td>
<td>+++</td>
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<td>OCTN1</td>
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<td>PEPT1</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PEPT2</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>SLCO Transporter</td>
<td></td>
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<tr>
<td>OATP1A2</td>
<td>–</td>
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<tr>
<td>OATP1B3</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>OATP2B1</td>
<td>–</td>
<td>+++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>PGT (OATP2A1)</td>
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</table>

The quantitative data of protein expression in Calu-3, BEAS2-B, and A549 represent protein expression by mass spectrometry (fmol/μg protein) in the present study: –, not detectable; +, low expression (1 ≤ fmol/μg protein > 0); ++, moderate expression (2 ≤ fmol/μg protein > 1); ++++, high expression (fmol/μg protein > 2). Data of Endter et al.\(^{11}\) represent intensity of gene expression by RT-PCR: –, no expression (0%–25% quartile of genes examined); +, low expression (25%–50% quartile); ++, moderate expression (50%–75% quartile); ++++, high expression (75%–100% quartile).

Data of Courcot et al.\(^{12}\) represent threshold cycle values (Ct) by Real time PCR: –, no expression (ΔCt>26); ..., very low (26≤ΔCt>24); +, low expression (24≤ΔCt>20); ++, moderate expression (20≤ΔCt>16); ++++, high expression (ΔCt≤16).

Tracheobronchial cells:

Calu-3

Trachea

Bronchi

Alveoli

BEAS2-B

Trachea

Bronchi

Alveoli

Alveolar type II cells:

Figure 1. Comparison of protein expression levels of selected transporters among (a) Calu-3 and primary cultured tracheal cells, (b) Calu-3 and primary cultured bronchial cells, (c) Calu-3 and primary cultured alveolar cells, (d) BEAS2-B and primary cultured tracheal cells, (e) BEAS2-B and primary cultured bronchial cells, (f) BEAS2-B and primary cultured alveolar cells, (g) NCI-H292 and primary cultured tracheal cells, (h) NCI-H292 and primary cultured bronchial cells, (i) NCI-H292 and primary cultured alveolar cells, (j) NCI-H441 and primary cultured tracheal cells, (k) NCI-H441 and primary cultured bronchial cells, (l) NCI-H441 and primary cultured alveolar cells, (m) A549 and primary cultured tracheal cells, (n) A549 and primary cultured bronchial cells, and (o) A549 and primary cultured alveolar cells. The quantitative data of primary cultured cells were cited from a previous study. The solid line passing through the origin represents the line of identity, and broken lines represent twofold differences. ULQ, under the LQ.
**Bronchiolar-alveolar cells:**


Because OATP2B1 was detected in primary cultured tracheal, bronchial, and alveolar cells, it is expected that it could be one of the useful transporter proteins for drug targeting to the lung or to achieve pulmonary drug absorption after inhalation.

However, OATP2B1 levels were below the limit of detection in all immortalized lung cell lines tested.

These findings suggest that an in vitro system of immortalized lung cell lines causes underestimation in the absorption in the pulmonary tract for OATP2B1 substrates.

The evaluation of transporter protein levels in advance of experiments is necessary to prevent the misinterpretation of their functional activities.
Further investigations are necessary to elucidate the similarity and disparity of transporters protein expression and cellular localization.

Possible factors to be considered

1. Culturing time (7 days → longer?)
   
mRNA expressions of MDR1, MRP5, MRP6, PEPT1 in Calu-3, and OAT4 were higher, and those of MRP4 and OCT3 were lower at day 15 compared with those at day 8. (Endter S, et al., J Pharm Pharmacol 61:583, 2009).

2. Culturing condition (liquid-covered culture(LCC) → air-interfaced culture (AIC))
   
Calu-3 cultured using air-interfaced culture (AIC) was more morphologically similar with airway epithelium than LCC. (Grainger CI, et al., Pharm Res 23: 1482, 2006).
Conclusion 1

This study appears to be the first to show the protein expression profiles of transporters in the lung.

We found that **OCTN1** and **MRP1** were highly expressed in the lung, which probably affected the distribution of inhaled drugs.

**OCTN1** was expressed at high levels in all regions, whereas **MRP1** showed regional differences in expression patterns, with higher expression in the bronchi and alveoli than in the trachea.

Interestingly, **OATP2B1** was expressed at the same level in all regions.

These findings may contribute to increased understanding of drug transport in human lung tissues.
Conclusion 2

**OCTN1** and **MRP1** protein expression in the plasma membrane fraction from five donors correlated well with the measured transport activity.

Therefore, measuring transporter expression in the plasma membrane fraction can be useful in understanding and predicting the transport of drugs after inhalation into the lungs.

Nevertheless, it is still challenging to predict the drug disposition of inhaled drugs because the cellular composition of the respiratory epithelium is considerably complicated based on the region.

It is also necessary to consider the characterization of inhaled drugs such as the particle size, density, velocity, and delivery devices.
Conclusion 3

The present study is the first to comprehensively characterize the protein expression levels of drug transporters in five commonly used immortalized cell lines.

There were similarities in the protein expressions of drug transporters between immortalized lung cell lines and primary cultured cells.

We also observed several gaps in protein amounts. Because these disparities can affect functional activities of drug transporters, protein expressions should be taken into consideration in the selection of appropriate immortalized lung cell lines.

These findings may contribute to the better understanding of drug transport in immortalized lung cell lines.
Future studies will be

1. Discovery of un-identified functional proteins

2. Polarized membrane localization of transporter/receptor/enzyme/channel protein expression

3. Regulation mechanism of transporter/receptor/enzyme/channel protein expression

4. Regulation mechanism of transporter/receptor/enzyme/channel protein localization and trafficking

5. Identification and quantification of drug target protein in the diseased lung

6. Discovery of biomarker protein of lung disease and drug response
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