Predicting Transporter-Based Drug Disposition and DDI using Quantitative Proteomics: Methodological Issue and Challenges

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Predicting Drug Disposition

• Predicting metabolic clearance from in vitro studies using PBPK models is reasonably accurate
• However…
  - Prediction of transporter mediated drug disposition is currently NOT possible because:
    • of lack of quantitative information on expression of transporters in human tissue important in drug disposition (e.g. liver, kidney)
    • Such data could also allow prediction of tissue drug concentrations and therefore efficacy and toxicity of drugs (e.g. hepatic).
**Tissue:Plasma Disconnect when Transporters are Involved**

- Typically we utilize plasma or blood concentrations to infer the pharmacokinetics of drugs at the site of efficacy or toxicity (e.g. hepatic)
- However, when active transporters are involved, there is a disconnect between the tissue and plasma/blood compartment
- This disconnect is exaggerated in the presence of DDI

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**Predicting Transporter –Based Drug Disposition & DDI**

Is there a way to predict disposition of drugs that are transported including tissue conc. when there is such tissue/plasma disconnect?

- Validated primary cells are currently not available for most tissues (e.g. BBB, intestines, kidneys).
- Where available (e.g. hepatocytes), predictions of transporter-based tissue PK has not been thoroughly validated.
**What Are Alternatives for Predicting Transporter-Based PK and DDI?**

**Hypothesis:** Scale up CL (including transporter-mediated) from transport studies in cells transfected with individual transporters

<table>
<thead>
<tr>
<th>In vitro CL</th>
<th>Scaling factors (SF)</th>
<th>In vivo CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLint via transport of interest in cell lines expressing the transporter</td>
<td>1. Transporter expression/g of tissue 2. Tissue weight</td>
<td>Contribution of individual transporter in tissue uptake/efflux</td>
</tr>
</tbody>
</table>

Scaling factors (SF) = \([T]_{in\ vivo}\) / \([T]_{in\ vitro}\)

**Challenges in Transporter Quantification by Immunoquantification**

- Pure transporter protein standards not available; therefore cannot compare expression of different transporters
- Lack of availability of antibodies; when available, poor selectivity
- Usually single or 2-3 protein quantification at a time; low throughput
- Semi-quantitative
University of Washington Research Affiliate Program on Transporters

- Goal is to quantify the expression of transporters (and interindividual variability) in various human tissues using targeted proteomics and LC/MS/MS

- Funded by a consortium: Merck & Co, Genentech, Biogen Idec (and Astra Zeneca) plus Gilead, Takeda, Ardea, BMS

What are the Elephants in the Room?

Current LC-MS/MS Methodology and Issues

- Tissue homogenization or washing of cell suspension
- Cell lysis and removal of soluble cytosolic proteins
- Selective isolation of membrane from the pellet
- Tryptic digestion of membrane proteins
- Reaction quenching and quantification

Internal standard does not address variability in sample preparation or trypsin digestion. Use labeled protein (SILAC) vs. labeled peptide (SIL) as an IS?

What are we measuring: plasma membrane or total membrane expression? Are we measuring functional protein (i.e. does expression correlate with activity)?

Is trypsin digestion complete?
**Questions**

  - Better questions to ask:
    - does transporter expression in cells and tissues track the expression of Na⁺-K⁺-ATPase (membrane marker) ?
- Is transporter activity correlated with transporter expression?
- Is trypsin digestion complete?
  - Is absolute protein quantification necessary or will absolute scaling factor suffice?

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**Isolation of Plasma Membrane from OATP2B1 expressing HEK293 cells (ab65400, Abcam)**

OATP2B1 tracks expression of Na⁺-K⁺-ATPase, suggesting that it is predominately in the plasma membrane

Kumar et al. DMD 2015
OATP1B1/2B1 transporter expression tracks Na⁺-K⁺ ATPase expression

Expression of OATP1B1 in Plasma Membrane Using Membrane Impermeable Biotinylating Reagent

Biotinylated fraction (Plasma membrane protein)

Kumar et al. DMD 2015
**OATP1B1 and BCRP: Activity-Expression Correlation (shRNA)**

Inhibitor: Estrone sulfate (1 µM)  
Substrate: Estradiol glucuronide (10 nM)

![Graph showing activity-expression correlation for OATP1B1 and BCRP](image)

\[ y = \frac{2.7}{8.1} \]
\[ R^2 = 1 \]

Inhibitor: KO143 (1.0 µM) + LY335984 (0.3 µM)  
Substrate: Hoechst 33342 (1.0 µM)

![Graph showing activity-expression correlation for BCRP](image)

\[ y = 1.1x \cdot 0.9 \]
\[ R^2 = 0.9 \]

**Only Vmax is affected, Km remained unchanged**

Kumar et al. DMD 2015

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**Is Trypsin Digestion Complete?**

- Possible to verify **ONLY** when purified protein is available
- Purified P-gp provided by Dr. Ambudkar of NCI
- The peptide LC/MS/MS approach was able to recover P-gp concentration within 73-125% accuracy
- These data indicate that trypsin digestion was complete and the method can accurately quantify the expression of P-gp
- But is confirming complete trypsin digestion necessary – no provided transporter expression in cell lines and tissue is measured using the same protocol?
Summary

- SIL and SILAC as internal standards are equivalent in quantification of transporter expression (provided digestion is optimized)
- Transporter expression in total membrane tracks that of Na⁺-K⁺-ATPase suggesting that the transporter measured is predominately expressed in the plasma membrane
- Biotinylation experiments suggest a plasma membrane vs. intracellular ratio of about 90:10
- Transporter activity and expression are correlated
- Absolute protein quantification is not necessary but absolute scaling factor is
- Collectively, the above data lend confidence to the use of cell lines for IVIVE of transporter-mediated drug clearance

Transporter expression in UW human liver bank tissue and in human hepatocytes
Relative Transporter Abundance Pie Chart

Wang et al. DMD 2015

Quantification of Human Hepatic Transporter Expression

Variability in OATP1B1 Expression is not Due to Age or Sex

Prasad et al. (Drug Metab Dispos 2013)

Genotype dependent OATP1B1 expression

- ↑ statin response and tolerance (Rodrigues et al., 2011, Donnelly et al., 2011)
- ↓ pravastatin AUC (Mwinyi et al., 2004)
Expression Data Predict Genotype-Dependent Changes in Repaglinide PK

Prasad et al. (Drug Metab Dispos 2013)

*1a/*1a ± 90% CI

*14/*14 ± 90% CI

32% ↓ in AUC₀⁻∞ of repaglinide in individuals with 388GG (Kalliokoski et al., 2008)

Pediatric Liver Samples

Tissue procurement (autopsy samples)
Children's Mercy Hospital, Kansas City
(Prof. Steve Leeder)

- Sex
- Ethnicity

Statistical analysis
ANOVA: Kruskal-Wallis test and Dunn's Multiple Comparison Test
Protein expression vs. age

Protein expression vs. age
Transporter expression in animal liver tissue and hepatocytes: comparison with human liver tissue

Transporter Abundance In Animal Livers

Cynomolgus
n=51-64
n=10

Beagle
n=6
n=16

Wang et al. DMD 2015
Summary

- These data provide a comprehensive picture of protein expression of hepatobiliary transporters in humans (adult and pediatric) and preclinical animal species.
- Data on interspecies variability in hepatobiliary transporter expression can help interpret and extrapolate preclinical PK and toxicity data from animals to humans.

Transporter expression in other human tissues
**Transporter Expression in Human Intestines**

N=6, 5 males, 1 female;

OATP1A2 could not be detected in the small intestine

**Interindividual Variability in Human Kidney (Cortex) Transporter Expression (N=14)**

- Expression of MATE2K and PEPT2 was below the lower level of quantification. BCRP could not be detected.
- Protein expression was not correlated with age or sex
Summary

• Quantitative proteomics is a promising technique for transporter quantification and has the potential to generate useful data to predict drug disposition in vivo including in tissues
• To validate such predictions, it would be ideal to have data on in vivo tissue distribution of drugs in humans
• Such data are best obtained using non-invasive imaging
• My laboratory has developed PET imaging methods to determine drug transport activity at various blood-tissue barriers (e.g. BBB, blood-placenta and in the liver)
**P-gp mediated $^{11}$C-Verapamil Efflux at the Blood-Brain Barrier in Humans**

Eyal et al., *Clin Pharmacol Ther.* 2010

Cb, cerebellum  
FC, frontal cortex  
OC, occipital cortex  
Pu, putamen  
Pf, pituitary  
T, thalamus  
V, lateral ventricle

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**P-gp Activity at the BBB is Reduced in Alzheimer’s Disease**

Regional cerebral blood flow (rCBF<sub>c</sub>)

Eyad et al., *J. Nucl. Med* '14

ERc as measured by $^{11}$C-Verapamil distribution
Hepatic Uptake and Biliary Excretion of $^{11}$C-Rosuvastatin in the Rat

Coronal 2 min SUV images of $^{11}$C-Rosuvastatin

Mol Pharm., '14
**11C-Rosuvastatin – Rifampin DDI in the Rat**

**11C-Rosuvastatin blood conc. – time profile**

AUCR\(_{0-15\text{min}}\)

\((\text{AUC}_{\text{RIF}}/\text{AUC}_{\text{control}})\)

\(\uparrow 230\%\)

**11C-Rosuvastatin hepatic conc. – time profile**

AUCR\(_{0-15\text{min}}\)

5\% \(\uparrow\)

Blood  Hepatocyte  Bile

ROS  BCRP/ MRP2  OATPs

He et al., Mol Pharm. 2014

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**Major Contributors**

Bhagwat Prasad  Anand Deo  Yang Yu  Jiake He  Li Wang

Vineet Kumar  Gabriela Patilea

UWRAPT members (Biogen, AZ, Genentech, Merck)
Other Collaborators

Dept. of Radiology: Drs. Jeanne Link, David Mankoff, Mark Muzi, Steve Shoner, Robert Miyako, Barbara Lewellen and the PET suite team

Dept. of Medicine: Ann Collier and her team

Univ. of Greifswald: Prof. Dr. Werner Siegmund, Dr. Stefan Oswald

Pfizer Inc: Drs. Yurong Lai and Larissa Balogh

NCI: Drs. Ambudkar and Shukla

OATP cells were generously provided by Drs. Sugiyama and Steiger

Acknowledgement: UWRAPT, NIH MH63641
Univ. of WA Health Sciences
SIL ≈ SILAC!

- OATP1B1
- OATP1B3
- OATP2B1
- P-gp

Prasad and Unadkat Int J Proteomics. 2014

Membrane isolation, OATP2B1 expressing HEK293 cells (Qproteome, Qiagen)

- Poor yield of plasma membrane

Kumar et al. DMD 2015
Peptide selection

1. For detection in mass spectrometry
   - Length between 6 and 16 amino acids
   - No posttranslational modifications
   - No genetic variation (unless interested in PGX)
   - No sequence conflicts
   - Moderate to high hydrophobicity

2. For complete digestion with trypsin
   - No transmembrane region

3. For stability of peptide
   - No methionine or cysteine residues
   - Contains leucine, isoleucine, valine, alanine or proline

4. Desirable secondary conditions
   - Length between 8 and 10 amino acids
   - No histidine or tryptophan
   - Sequence homology between species


Method Development

Surrogate peptide selection

1. Unique
2. Stable
3. Good MS response
4. Optimal LC retention
5. Digestion efficiency
6. Reproducibility

- Pink (highlight): transmembrane region
- Green font = Known SNP
- Blue (highlight) = PTM
- Red (highlight) = RR, KK, RK or KR
- Grey (highlight) = Sequence conflict
- Underlined, italicized = M, C, H and W
- Yellow (highlight) = Ideal peptides

Prasad and Unadkat AAPS J., 2014
**Plasma and Brain $^{11}C$-verapamil radioactivity concentration-time profiles in the absence and presence of CsA**


**PK of $^{11}C$-Rosuvastatin in the Rat**

Table 1. Pharmacokinetic parameters of $^{11}C$-ROS as estimated by the five-compartment model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>RIF-treated</th>
<th>RIF-treated / Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>331±15</td>
<td>335±10</td>
<td>100.0±0.5 **</td>
</tr>
<tr>
<td>CL (L/min)</td>
<td>1.90±0.09</td>
<td>2.06±0.10</td>
<td>108±0.1 **</td>
</tr>
<tr>
<td>CL (L/min)</td>
<td>0.40±0.08</td>
<td>0.43±0.08</td>
<td>107±0.04 **</td>
</tr>
<tr>
<td>CL (L/min)</td>
<td>1.74±0.37</td>
<td>1.71±0.37</td>
<td>100±0.1 **</td>
</tr>
<tr>
<td>CL (L/min)</td>
<td>2.04±0.22</td>
<td>2.07±0.22</td>
<td>101±0.1 **</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>23.3±1.5</td>
<td>23.3±1.5</td>
<td>100±0.1 **</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>11.1±0.9</td>
<td>10.2±0.9</td>
<td>92.7±0.1 **</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>6.8±0.2</td>
<td>6.7±0.2</td>
<td>98.5±0.1 **</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>2.2±0.1</td>
<td>2.2±0.1</td>
<td>100±0.1 **</td>
</tr>
</tbody>
</table>

Data are mean = S.D. (n=3). The value in parentheses represents % CV in parameter estimates. *p<0.05, **p<0.01 when compared with control rats.
### Signature peptides

<table>
<thead>
<tr>
<th>Transporters</th>
<th>Species</th>
<th>No. of peptides ordered</th>
<th>No. of peptides applied</th>
<th>Reason for using &lt;2 peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcrp</td>
<td>D, M, and R</td>
<td>3</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>BSEP/Bsep</td>
<td>H, D, M, and R</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>MATE1/Mate1</td>
<td>H</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Mdr1</td>
<td>D, M, and R</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Mdr2</td>
<td>D and M</td>
<td>4</td>
<td>1</td>
<td>Poor MS Response/discrepancy</td>
</tr>
<tr>
<td>MRP3/Mrp3</td>
<td>D, and R</td>
<td>2</td>
<td>2</td>
<td>Poor MS Response</td>
</tr>
<tr>
<td>MRP4/Mrp4</td>
<td>H, D, and M</td>
<td>2</td>
<td>1</td>
<td>Poor MS Response</td>
</tr>
<tr>
<td>NTCP/Ntcp</td>
<td>D</td>
<td>2</td>
<td>0</td>
<td>Poor MS Response</td>
</tr>
<tr>
<td>Oatps</td>
<td>D, M, and R</td>
<td>2</td>
<td>1</td>
<td>Poor MS Response/discrepancy</td>
</tr>
<tr>
<td>OCT1/Oct1</td>
<td>H, D, M, and R</td>
<td>2</td>
<td>1</td>
<td>Poor MS Response</td>
</tr>
</tbody>
</table>

H: human; D: dog; M: monkey; R: rat; Peptides labeled in red were used for transporter quantification among species.
Hepatobiliary clearance of the drug

- OATP1B1
- OATP1B3
- OATP2B1
- CYPs
- MRP2
- UGTs
- BCRP

Sandwich-cultured hepatocytes

Used to predict transporter-mediated hepatic clearance

Problems
- High cost
- Low-throughput
- Time-varying expression of transporter
- Hard to apply this technique for the prediction of drug disposition in other tissues
Interspecies Comparison of Abundance of Hepatobiliary Transporters

• Sinusoidal transporters

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>D</th>
<th>M</th>
<th>SD</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Sample size</td>
<td>55</td>
<td>6</td>
<td>10</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Hepatocytes Sample size</td>
<td>NA</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

H: human; D: dog; M: monkey; SD: Sprague-Dawley rat; W: Wistar rats
ND: Not determined; BLQ: Below LLOQ
***: p <0.001; **: p <0.01; *: p <0.05

Interspecies Comparison of Abundance of Hepatobiliary Transporters

• Canaliccular transporters

<table>
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</tr>
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H: human; D: dog; M: monkey; SD: Sprague-Dawley rat; W: Wistar rats
ND: Not determined; BLQ: Below LLOQ
*: p <0.05; **: p <0.01; ***: p <0.001
**Biotinylation Experimental Procedure**

1. Biotinylate cells, 45 min, 4°C
2. Quench reaction and harvest cells
3. Centrifuge and dissolve the pellet in lysis buffer (lysate)
4. Incubate the lysate on column containing NeutrAvidin resin
4a. Collect non-biotinylated protein fraction as centrifugate
4b. Collect biotinylated protein fraction as centrifugate
5. Incubate the column containing NeutrAvidin resin with elution buffer
6. Collect biotinylated protein fraction as centrifugate
7. Trypsin digestion
8. LCMS analysis

**Intestinal Expression of ABC Transporters**

N=6; 5 males and 1 female

P values refer to difference in expression between small intestine and colon

- **ABCB1**
  - Protein expression (pmol/mg)
  - p < 0.001

- **ABCC2**
  - Protein expression (pmol/mg)
  - p = 0.085

- **ABCC3**
  - Protein expression (pmol/mg)
  - p < 0.001

- **ABCG2**
  - Protein expression (pmol/mg)
  - p < 0.001
Intestinal Expression of SLC Transporters

- OATP2B1
  - Protein expression (pmol/mg)
  - p = 0.339

- OCT1
  - Protein expression (pmol/mg)
  - p = 0.002

- OCT3
  - Protein expression (pmol/mg)
  - p = 0.002

- PEPT1
  - Protein expression (pmol/mg)
  - p < 0.001

- ASBT
  - Protein expression (pmol/mg)
  - p < 0.001

N=6, 5 males, 1 female;
P values refer to difference in expression between small and large intestine

No intestinal expression of OATP1A2 observed (mRNA or protein)

Biotinylation study with OATP1B1 expressed CHO cells @ 4°C

Biotinylation reagent: 625%

*Total recovery of marker proteins compared to lysate was 92.1 – 102.3%
Biotinylation of OATP1B1 expressed in CHO cells at 4°C

Expt. 1
Expt. 2

2.4 mg/10mL: (100%)

*Total recovery of marker proteins compared to lysate in expt. 1 and 2 was 85.4 – 107.6% and 87.3 – 115.5% respectively

Immunohistochemistry evidences indicate that OATP1B1 and other transporters are predominantly localized in the plasma membrane of the human liver

Human Liver

Mol Pharmacol 68:1031–1038, 2005
**Is Absolute Transporter Quantification Necessary?**

- **No, but, absolute scaling factor (ASF) is necessary**
- Provided that the same methodology is used for quantification of protein in cell lines vs. tissue, ASF is valid even if
  - trypsin digestion is not complete
  - extraction and recovery of protein from the membrane is not complete
  - as long as there is consistency in the above between cells and tissues

\[ SF = \frac{[T]_{\text{in vivo}}}{[T]_{\text{in vitro}}} \]

**ASF Cells vs. Tissue**

<table>
<thead>
<tr>
<th></th>
<th>Peptide 1</th>
<th>Peptide 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT2 (pmol/mg) in OCT2-HEK293 cells</td>
<td>21.23</td>
<td>36.3</td>
</tr>
<tr>
<td>OCT2 (pmol/mg) in human kidney tissue</td>
<td>4.58</td>
<td>7.05</td>
</tr>
<tr>
<td>Scaling factor (ASF)</td>
<td><strong>0.2</strong></td>
<td><strong>0.2</strong></td>
</tr>
</tbody>
</table>