In Vitro Models for Quantitative Prediction of Hepatobiliary Clearance

Kim L. R. Brouwer, PharmD, PhD

William R. Kenan, Jr., Distinguished Professor
Associate Dean for Research and Graduate Education
UNC Eshelman School of Pharmacy,
Curriculum in Toxicology,
The University of North Carolina at Chapel Hill
Conflict of Interest Statement

Dr. Brouwer is co-inventor of the sandwich-cultured hepatocyte technology for quantification of biliary excretion (B-CLEAR®) and related technologies, which have been exclusively licensed to Qualyst Transporter Solutions.
Hepatobiliary Clearance

- **Cl\textsubscript{biliary}** may contribute significantly to **Cl\textsubscript{hepatic}**
- Altered **Cl\textsubscript{biliary}** due to genetics, disease states or drug interactions may affect pharmacological efficacy and/or toxicity (systemic, hepatic and/or intestinal)
Hepatic Uptake and Efflux Transporters

(Adapted from Ho and Kim, Clin Pharmacol Ther, 78:260, 2005)
Selection of the appropriate model system depends on the question. What parameter(s) do you need to predict?

- Hepatic uptake
- Hepatic clearance
- Biliary excretion (extent)
- Biliary clearance
- Hepatocyte accumulation
- Hepatotoxicity
- Hepatic transporter involvement and interactions
Modeling & Simulation

Throughput

Membrane Vesicle System

<table>
<thead>
<tr>
<th>Models</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Membrane Vesicle System</strong></td>
<td>ATP</td>
</tr>
<tr>
<td></td>
<td>AMP</td>
</tr>
<tr>
<td></td>
<td><strong>Net-ATP dependent</strong></td>
</tr>
<tr>
<td>transfection</td>
<td>ATP</td>
</tr>
<tr>
<td>ATP</td>
<td>Endogenous Transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>Transporter of interest</td>
</tr>
<tr>
<td>ATP</td>
<td>Control-transfected</td>
</tr>
<tr>
<td>ATP</td>
<td>Endogenous Transporter</td>
</tr>
</tbody>
</table>

**Transfected Cells**

**Suspension Hepatocytes**

<table>
<thead>
<tr>
<th>Methods</th>
<th>suspended Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase perifusion</td>
<td>Hepatocytes isolation</td>
</tr>
<tr>
<td>Hepatocyte isolation</td>
<td>Percoll gradient</td>
</tr>
<tr>
<td>Hepatocytes + radioactive compound</td>
<td>Dextrose centrifugation</td>
</tr>
<tr>
<td>Hepatocytes + radioactive compound</td>
<td>Cut</td>
</tr>
<tr>
<td>Hepatocytes + radioactive compound</td>
<td>Radioactivity remaining</td>
</tr>
<tr>
<td>Hepatocytes + radioactive compound</td>
<td>Bile taken up by the hepatocytes</td>
</tr>
</tbody>
</table>

**Sandwich-Cultured Hepatocytes**

<table>
<thead>
<tr>
<th>Methods</th>
<th>sandwich-Cultured Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>overlay 16 h after plating</td>
</tr>
<tr>
<td>Day 1</td>
<td>change medium daily</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
</tr>
</tbody>
</table>

**Isolated Perfused Liver**

<table>
<thead>
<tr>
<th>Methods</th>
<th>isolated Perfused Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inferior Vena Cava Outflow</td>
<td>Gal Bladder</td>
</tr>
<tr>
<td>Portal Vein Inflow</td>
<td>Bile Duct</td>
</tr>
</tbody>
</table>

Yang...Brouwer, *Transporters in Drug Development*; Chapter 9, Springer 2013
In Vitro Methods to Support Transporter Evaluation in Drug Discovery and Development

KLR Brouwer¹, D Keppler², KA Hoffmaster³, DAJ Bow⁴, Y Cheng⁵, Y Lai⁵, JE Palm⁶, B Stieger⁷ and R Evers⁸; on behalf of the International Transporter Consortium

This white paper addresses current approaches and knowledge gaps concerning methods to assess the role of transport proteins in drug/metabolite disposition in humans. The discussion focuses on in vitro tools to address key questions in drug development, including vesicle- and cell-based systems. How these methods can be used to assess the liability of compounds for transporter-based drug–drug interactions (DDIs) in vivo is also explored. Existing challenges and approaches to examine the involvement of transporters in drug disposition are discussed.

- Overview of experimental systems currently employed to conduct in vitro transporter studies
- Applications, strengths and limitations of each system
- Issues concerning data interpretation
- Integration of in vitro transporter data to address important questions in drug discovery and development

Brouwer et al., Clin Pharmacol Ther, 94:95, 2013
Recombinant Cell Lines Expressing Uptake Transporters

- **Applications**
  - Evaluate drug interactions with uptake transporters (i.e., OATPs, OCTs)
  - Determine substrate specificity and identify inhibitors

- **Requirements**
  - Low endogenous transport activity in cell line

![Diagram of process]

1. Plasmid DNA
2. Transfection
3. Cells expressing transporter(s)
Bidirectional Transport in Recombinant Polarized Cell Monolayers

Cells expressing transporter(s) → Transfection → Cells expressing transporter(s)

Apical (A) → Basolateral (B)

Drug transport → Time

Cui et al., Mol Pharmacol, 60:934, 2001
Bidirectional Transport in Recombinant Polarized Cell Monolayers

- **Strengths**
  - Transport is less influenced by nonspecific binding because only the compound crossing the cell monolayer is measured
  - Suitable to assess active transport vs. diffusion
  - Stably transfected cell lines can be passaged for multiple use or cryopreserved

- **Limitations**
  - Generation/characterization of stable recombinant cell lines is time consuming
  - Transporter expression levels vary between labs
  - Endogenous transporter activity may confound data interpretation
  - Mass balance needs to be assessed
  - Complicated kinetic studies
Membrane Vesicle-Based Transporter Assays for Efflux Transporters

- Inside-out oriented membrane vesicles prepared from:
  - Baculovirus-infected insect (Sf9 or Sf21) cells
  - Drug-selected cell lines
  - Stably or transiently transfected cell lines (HeLa, HEK293, MDCK)
  - Purified basolateral or apical plasma membranes from organ of interest
Membrane Vesicle-Based Transporter Assays

- **Strengths**
  - Good for compounds with low permeability
  - Cytotoxic compounds don’t impact experimental system
  - High transporter expression levels in recombinant systems
  - Large batches can be prepared and cryopreserved
  - Able to preload with buffers and/or substances
  - Substrates in incubation buffer have direct access to active site

- **Limitations**
  - Not suitable for compounds with high permeability or high nonspecific binding
  - Hypoglycosylation in insect cells (e.g., Sf9) may alter transport
  - Endogenous transport activity in expression system may confound data interpretation
  - Transporter activity varies from batch to batch
In Vitro Assays: Non-Hepatocyte Systems

- **Strengths**
  - High-throughput
  - Readily available
  - Common approach to determine whether a substrate *can* be transported by a specific protein

- **Limitations**
  - May not accurately predict which proteins predominantly transport substrates *in vivo* in the hepatocyte
  - Transport proteins are not expressed at physiologically relevant levels – IVIVE is challenging
  - Not a holistic system with all relevant transporters, endogenous compounds, regulatory machinery, metabolic capacity, intracellular compartments and binding proteins
  - Limited ability to assess complex drug-transport interactions (e.g., non-competitive mechanisms, metabolite interactions)
Suspended Hepatocytes

Collagenase perfusion → Hepatocyte isolation → Percoll gradient

Hepatocytes + Radioactive compound ± Inhibitor

Centrifugation

Silicone oil

KOH

Remaining radioactivity

Cut

Radioactivity taken up by the hepatocytes
Suspended Hepatocytes

- Suitable for measuring *initial* hepatic uptake and transporters involved in initial substrate uptake
- *Not* suitable for measuring biliary clearance
  - Canalicular proteins are not properly localized

Immunohistochemical localization of P-gp (C219) and Oatp1a1 in freshly isolated rat hepatocytes. Single frames from 3D reconstruction of z-stack (1 µm slices)

A. Oatp1a1 (red)  
B. P-gp (green)  
C. Composite image

Bow...Brouwer, *Drug Metab Dispos*, **36**:198, 2008
Suspended or Plated Hepatocytes

**Strengths**
- Cryopreserved or freshly isolated hepatocytes from the species of interest
- Human hepatocytes can be pooled to minimize interindividual variability
- Expression of uptake transporters is relatively close to *in vivo*
- Allows assessment of contribution of multiple hepatic uptake transporters simultaneously
- Allows assessment of active uptake vs. passive diffusion

**Limitations**
- Loss of cell polarity
- Minimal functional activity of canalicular efflux transporters; basolateral efflux transporters are functional
- Rapid loss of metabolic activity in culture
Sandwich-Cultured Hepatocytes

Day 0

Overlay ~20 h after plating

Day 1

change medium daily

Day 4 (Rat)

Day 7 (Hu)

Morphology

CDF Localization

Swift et al. Drug Metab Rev 42:446, 2010
CDF: 5-(6)-carboxy-2',7'-dichlorofluorescein

Standard Buffer

Ca²⁺-free Buffer

Substrate in Bile Canaliculi

B-CLEAR® technology is covered by US Pat. No. 6,780,580 and other US and International patents, both issued and pending, and is exclusively licensed to Qualyst Transporter Solutions
Experimental Parameters:

**Biliary Excretion Index (%)**

\[ \text{Biliary Excretion Index} = \left( \frac{\text{Total Accumulation} - \text{Cellular Accumulation}}{\text{Total Accumulation}} \right) \times 100 \]

**In Vitro Biliary Clearance**

\[ \text{In Vitro Biliary Clearance} = \frac{\text{Total Accumulation} - \text{Cellular Accumulation}}{AUC_{\text{media}}} \]

**Intracellular Concentration**

\[ \text{Intracellular Concentration} = \frac{\text{Cellular Accumulation}}{\text{Volume}_{\text{intracellular}}} \]

B-CLEAR® is covered by US Pat. No. 6,780,580 and other US and International patents both issued and pending.
Sandwich-Cultured Hepatocytes

● **Strengths**
  - Normal cell polarity re-established
  - Biliary clearance can be measured
  - Holistic system expressing uptake and efflux transporters, metabolic enzymes and regulatory machinery
  - Cryopreserved or freshly isolated hepatocytes from the species of interest can be used
  - Suitable to identify transport inhibitors (both competitive and non-competitive) and inducers
  - Demonstrated *in vitro-in vivo* correlations between preclinical species and humans

● **Limitations**
  - Requires time in culture for proper localization of transporters
  - Less suitable for low-clearance compounds
  - Enzyme/transporter expression/activity may be modulated by culture conditions
What is the Relevant Concentration for Accurate Risk Assessment?

- Relevant concentrations: total vs unbound extracellular vs intracellular?
- Should protein be included in the incubation medium?
- How do we extrapolate IC$_{50}$ data from *in vitro* systems (e.g., vesicle-based transporter assays) to *in vivo*?

Mechanisms of Hepatotoxicity: Direct and Indirect Interactions with Bile Acids

- BSEP (Bile Salt Export Pump)
- NTCP (Sodium-Taurocholate Cotransporting Polypeptide)
- MRP (Multidrug Resistance–Associated Protein)
- OST (Organic Solute Transporter)

Changes in intracellular bile acid concentrations and composition
Changes in serum bile acid concentrations and composition

Nuclear hormone receptor regulation of bile acid synthesis and transport
Post-translational modifications of bile acid transporters

Hepatotoxicity
Essential Components of a Predictive Hepatic Model for Risk Assessment:

✓ Uptake
  • Sinusoidal uptake transport proteins

✓ Efflux
  • Biliary and/or basolateral efflux transport proteins

✓ Metabolism
  • Metabolic enzymes for elimination

✓ Regulation
  • Induction of transport and metabolism

The intracellular concentration determined in sandwich-cultured hepatocytes is a function of all of these processes

Generation of in vivo relevant intracellular concentrations using B-CLEAR® hepatocytes

Courtesy of Qualyst Transporter Solutions
Recommendations

- Numerous *in vitro* assays are available to predict hepatic uptake and the involvement of transporters in hepatobiliary clearance.

- Assays must be selected based on the compound characteristics and the question(s) that need to be answered; assay limitations always must be considered.

- Sandwich-cultured hepatocytes are the only *in vitro* model that can accurately predict biliary clearance for IVIVE.

- Additional research is needed to characterize the assay conditions (e.g., protein, bile acids, other media additives) and concentration(s) (e.g., unbound, total) that are most predictive for IVIVE, cellular regulatory mechanisms that impact hepatobiliary clearance, and whether more complex models are able to improve prediction accuracy.
Acknowledgments

International Transporter Consortium
Methods Whitepaper Coauthors:
Raymond Evers (Merck)
Dietrich Keppler (German Cancer Institute)
Keith Hoffmaster (Novartis)
Dan Bow (AbbVie)
Yaofeng Cheng (Bristol-Meyers Squibb)
Yurong Lai (Bristol-Meyers Squibb, Pfizer)
Johan Palm (AstraZeneca)
Bruno Stieger (Univ. Hospital, Zurich)

- National Institutes of Health
  R01 GM41935, M01 RR00046 and T32 ES007126
- Deutsche Forschungsgemeinschaft
  Grant Ko4186/1-1
- UNC Royster Society of Fellows
- Amgen Predoctoral Fellowships

Brouwer Lab
Dan Bow
Giulia Ghibellini
Kathleen Köck
Xingrong Liu
Nathan Pfeifer
Brandon Swift
Kyunghee Yang