Strengths and Limitations of In Vitro Xenobiotic Metabolism Assays

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In Vitro Models to Study Drug Metabolism

- Recombinant expressed enzymes
- Hepatic cell lines overexpressing metabolizing enzymes
- Subcellular fractions: microsomes/S9
- Differentiated hepatic cell lines
- Primary hepatocytes
  - Suspensions
  - Sandwich cultures
  - ‘NextGen’ culture models
- Liver Slices
- Isolated perfused organs

Complexity, Metabolic Pathway Coverage & Physiological Relevance
What is the ‘Metabolism’ Question?

• Will a chemical be appreciably metabolized into alternate chemical structures? How much and how do we translate quantitatively to in vivo?
  – Primary hepatocytes, liver S9, liver microsomes, recombinant enzymes
  – IVIVE approaches to predict pharmacokinetics (i.e. $C_{\text{max}}$, $C_{\text{ss}}$, $T_{1/2}$, etc...)

• What metabolic pathways are likely involved in clearance (reaction phenotyping)?
  – Recombinant enzymes, liver microsomes/S9 with inhibitors or poor metabolizers

• What metabolite(s) will form and at what relative quantities/proportions?
  – Primary hepatocyte suspensions, liver microsomes/S9

• Is metabolic activation to toxic metabolites a potential issue for a chemical?
  – Liver S9 (e.g. Ames test for mutagenicity), primary hepatocytes (i.e. protein adducts, GSH conjugates), P450 over-expressing cell lines

• Will a chemical inhibit metabolism (i.e. TDI) that alters drug/chemical clearance?
  – Liver microsomes, primary hepatocyte suspensions

• Will chemical induce liver enzymes that alters drug/chemical clearance?
  – Primary hepatocyte sandwich cultures, HepaRG cultures
In Vitro Liver Models Derived from Human Liver Most Phys. Relevant
‘Full’ metabolic competence is NOT an intrinsic property of primary hepatocytes, but dependent on culture environment.
Comparison of Metabolic Competencies

- Metabolic competence/proportions not intrinsic, subject to environment
- 3D & flow models can greatly improve metabolic competence
- Vast majority of metabolic clearance assays in suspensions of PHHs

Jackson et al., submitted, Drug. Metab. Disp.
Metabolic Stability Assays (Substrate Depletion)

**Assay conditions summary:**
- 1.2 mL initial reactions
- WEM with ITS⁺ & HEPES buffer
  - ITS⁺: insulin, transferrin, selenous acid, BSA (1.25 g/L), and linoleic acid
- 120 rpm (orbital)
- Assay suspension aliquots crashed in ACN (1:1)
- Monitor viability (e.g. Trypan Blue)

~35-50 g/L albumin in human blood (35X in vitro levels used here)
Intrinsic Clearance $CL_{int}$

The Prediction of Human Pharmacokinetic Parameters from Preclinical and *In Vitro* Metabolism Data

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**IN VIVO CLEARANCE OF ETHOXYCOUMARIN AND ITS PREDICTION FROM IN VITRO SYSTEMS**

Use of Drug Depletion and Metabolite Formation Methods in Hepatic Microsomes and Isolated Hepatocytes

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\[
CL_{int} = \frac{0.693}{t_{1/2}} \times \frac{\text{g liver wt}}{\text{kg body wt}} \times \frac{\text{ml incubation}}{\text{cells per incubation}} \times \frac{1.35 \times 10^6 \text{ cells}}{\text{g liver wt}}
\]

For a first order reaction with $[S] \ll K_m$, the $t_{1/2}$ values should be constant.
Metabolic Stability with ToxCast (Phase I & Phase II) Chemicals

Pooled Suspensions of Primary Hepatocytes from >10 donor preps

Collaboration between The Hamner, EPA, and Life Tech

Assay conditions summary:
- 100 µL assays
- 1 & 10 µM concentrations
- WEM with ITS+ & HEPES buffer
  - ITS+: insulin, transferrin, selenous acid, BSA (1.25 g/L), and linoleic acid
- 300 rpm (orbital)
- Assay suspension aliquots crashed in ACN (1:1)
- Monitored viability (e.g. Trypan Blue)
- Heat-treated & no-cell controls included to assess non-specific binding/recovery
Strengths of Suspension Hepatocyte $CL_{int}$ Approach:

- Suspension hepatocytes historically proven to be a useful model system, superior to microsomes/S9 due to Phase II metabolism capacity, de novo cofactor synthesis
- Suspension hepatocytes support a broad complement of transformation types and active uptake transport
- Suspension hepatocytes represent in vivo-like levels/proportions of metabolic competence
- Monitoring loss of parent captures broad complement of metabolism pathways
- Crashing cells & supernatants to maximize recovery of parent chemical can improve mass balance
- Useful $CL_{int}$ predictions obtained, largely in agreement with in vivo metabolic clearance (within ~2-3 fold)

Limitations of the Suspension Hepatocyte $CL_{int}$ Approach Used to date:

- Suspensions of hepatocytes are short lived (~2-4 hrs)
- Suspensions hepatocyte metabolic competence half-lives ~1-2 hrs limiting effectiveness with lower turnover compounds that could also alter metabolite profile outcomes
- Physiological levels of serum proteins (i.e. albumin) were not used during incubations (~30-fold lower)
- Crashing cells and supernatants together limits ability to understand partitioning kinetics and the impact of uptake transport
- Suspension hepatocytes thought to be devoid of canalicular efflux transport and limited in basolateral efflux that may alter metabolism outcomes
Suspension PHHs Often Ineffective with Lower Turnover Compounds?
Temporal Effects on CYP1A2 & CYP3A Activities in Suspension vs. Plated Cultures

IT50 = 2.49 h ± 0.53

IT50 = 21.3 h ± 2.1

IT50 = 1.63 h ± 0.08

IT50 = 28.8 h ± 20.4

CL_{int} in ‘Early’ Cultures of PHHs

Collagen-I coated, plated pre-wetted with 60 µL plating media

Plate Cryo Heps @ 50,000 cells/well

Remove spent culture media & Add cmpds 1 µM final in WEM with HEPES

Allow attachment for ~4 hrs

Incubate for T=0, 0.25, 1, 2, 6, 18, 24, and 48 hrs

Collect supernatants, flash freeze sups & plates for analytical

ACN crash & analytical on sups & cell fractions

Collaboration-BI
Assay Optimization of $\text{CL}_{\text{int}}$ in ‘Early’ Cultures of PHHs

Initial Attachment 48 hrs, 200 RPM 48 hrs, 800 RPM

CYP3A4/5 (OHMDZ) Activity (pmol/min/million cells)

ATP (RLU)

No Shaking 200 RPM 200+OL 800 RPM Delayed

No Shaking 200 RPM 200+OL 800 RPM Delayed
Results with ‘Early’ PHH Cl\textsubscript{int} Model

- Extended to a broader set of ~30 chemicals
- Began evaluation of pooled plateable approach
- Overlay had little effect on Cl\textsubscript{int} within 48hrs evaluated

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class</th>
<th>In vivo Cl\textsubscript{non-renal, obs}</th>
<th>% Q\textsubscript{h}</th>
<th>In vitro CL\textsubscript{h} (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Warfarin</td>
<td>Acidic</td>
<td>0.1</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>Acidic</td>
<td>0.4</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>S-Mephenytoin</td>
<td>Basic</td>
<td>0.5</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>Neutral</td>
<td>0.8</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Neutral</td>
<td>1.1</td>
<td>5</td>
<td>2.1</td>
</tr>
<tr>
<td>Clozapine</td>
<td>Basic</td>
<td>2.9</td>
<td>14</td>
<td>3.4</td>
</tr>
<tr>
<td>Triazolam</td>
<td>Neutral</td>
<td>4.7</td>
<td>23</td>
<td>1.7</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Neutral</td>
<td>4.9</td>
<td>24</td>
<td>3.6</td>
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<tr>
<td>Zolpidem</td>
<td>Neutral</td>
<td>5.7</td>
<td>28</td>
<td>2.1</td>
</tr>
<tr>
<td>Accuracy</td>
<td>% within 2-fold</td>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>% within 3-fold</td>
<td></td>
<td></td>
<td>89</td>
</tr>
</tbody>
</table>
‘NextGen’ In Vitro Liver Models to Predict Metabolic Clearance

- Vastly improved longevity over suspensions of PHHs
- Maintain levels of metabolic competence exceeding 4-day cultures of PHHs (< suspension PHHs)
- Appear useful for multiple questions including:
  - $\text{CL}_{\text{int}}$
  - Metabolite formation
  - Metabolite profiling
  - Chemical accumulation

### Table 7

<table>
<thead>
<tr>
<th>Method</th>
<th>Hepatic Clearance of Faldaprevir at: 120 mg QD</th>
<th>Hepatic Clearance of Faldaprevir at: 240 mg QD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well stirred</td>
<td>9.37</td>
<td>8.08</td>
</tr>
<tr>
<td>Well stirred + uptake</td>
<td>31.1</td>
<td>22.9</td>
</tr>
<tr>
<td>Poulin method</td>
<td>49.4</td>
<td>42.7</td>
</tr>
<tr>
<td>CL/F observed</td>
<td>67.4</td>
<td>19.7</td>
</tr>
<tr>
<td>In vivo CL (F = 0.5–1)</td>
<td>33.7–67.4</td>
<td>9.85–19.7</td>
</tr>
</tbody>
</table>
Xenobiotic Metabolism Competence Mapping

- Develop xenobiotic metabolism assay suite
  - Liver enzyme specific activity panel (& mRNA)
  - Metabolite profiles over time (with targeted ID)
  - Metabolic clearance ($Cl_{INT}$) capacities

- Evaluate xenobiotic metabolism ‘competence’ in various in vitro models representing adult liver, pediatric liver, Tox21 assays at various states of cellular differentiation

- Contextualize xenobiotic metabolism competence, grounded in metabolite profiles over time, that collectively define metabolic competence states

- Deploy physiologically-relevant (i.e. levels/proportions) xenobiotic metabolism to in vitro toxicology research
  - direct, conditioned chemicals, co-cultures, flow systems

- Link parent and metabolite chemical structures to phenotypic (high content imaging) and high resolution ‘omics responses (i.e. transcriptomics)


<table>
<thead>
<tr>
<th>Age group</th>
<th>Low clearance chemicals</th>
<th>High clearance chemicals</th>
<th>High + intermediate clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premature neonates</td>
<td>not available</td>
<td>3.34 ± 1.27</td>
<td>4.18 ± 1.26</td>
</tr>
<tr>
<td>Full-term neonates</td>
<td>3.40 ± 2.18$^a$</td>
<td>1.99 ± 0.67</td>
<td>2.38 ± 0.59</td>
</tr>
<tr>
<td>1 week–2 months</td>
<td>4.34 ± 0.62$^a$</td>
<td>1.85 ± 0.38</td>
<td>1.96 ± 0.41</td>
</tr>
<tr>
<td>2–6 months</td>
<td>1.25 ± 0.31</td>
<td>0.90 ± 0.26</td>
<td>0.94 ± 0.28</td>
</tr>
<tr>
<td>6 months–2 years</td>
<td>0.57 ± 0.16</td>
<td>0.26 ± 0.12</td>
<td>0.52 ± 0.14</td>
</tr>
<tr>
<td>2–12 years</td>
<td>0.60 ± 0.11</td>
<td>0.72 ± 0.24</td>
<td>0.72 ± 0.10</td>
</tr>
</tbody>
</table>

http://depts.washington.edu/sfields/metabolites/

- Suspension PHHs
- Hepatopac
- Tox21 cell lines
- Stem cells (i.e. iPSCs)

Cell biology phenotypes (i.e. proliferation) → ‘omics/ pathways

Example metabolite profile

http://depts.washington.edu/sfields/metabolites/
In Silico Models for Reverse TK IVIVE When No In Vitro Data Are Available

Nisha Sipes Compiled 28 Tox21 cmpds
- *in silico* parameters (Fub, CLint)
- *in vitro* parameters (Fub, CLint)
- *in vivo* measured $C_{\text{max}}$ values (human, Drug Matrix)

In Silico CL$_{\text{int}}$ Models
- CYP1A2
- CYP2C9
- CYP2C19
- CYP2D6
- CYP3A4

Weighted sum by relative expression levels in human liver
In Silico Models for Reverse TK IVIVE When No In Vitro Data Are Available

Nisha Sipes

- in silico parameters (Fub, CLint)
- in vitro parameters (Fub, CLint)
- in vivo measured Cmax values (human, Drug Matrix)

Accepted Manuscript

Title: QSARs for estimating intrinsic hepatic clearance of organic chemicals in humans

Author: Alessandra Pirovano Stefan Brandmaier Mark A.J. Huijbregts Ad M.J. Ragas Karin Veltman A. Jan Hendriks

PII: S1382-6689(16)30017-5
DOI: http://dx.doi.org/doi:10.1016/j.etap.2016.01.017
Reference: ENVTOX 2442

To appear in: Environmental Toxicology and Pharmacology

Received date: 8-9-2015
Revised date: 19-1-2016
Accepted date: 21-1-2016
How Do We Apply These In Vitro to In Vivo Translation Approaches to Tox21 Where Metabolism Data is Not Available?

**IVIVE**

- **Chemical Assay**
  - $AC_{50}$
  - $E_{max}$

- **Chemical Biomarker**
  - $C_{max}$
  - Efficacy

**In Vivo Data**
- In Vitro $CL_{int}$, fub predictions
- In Silico $CL_{int}$, fub predictions

Please visit Nisha’s Poster!

**Computational Models to Correlate In Vitro to In Vivo Activity**

Sipes\(^1\) NS, Wambaugh\(^2\) JF, Pearce\(^2\) R, Wetmore\(^3\) BA, DeVito\(^1\) MJ, Auerbach\(^1\) SS, Ferguson\(^1\) SS

\(^1\)NTP/NIEHS/NIH, \(^2\)NCCT/ORD/EPA, \(^3\)The Hamner Institutes for Health Sciences, Research Triangle Park, NC 27709
Summary

• Effective in vitro models to predict in vivo metabolism generally employ in vivo-like levels of xenobiotic metabolism competence

• The rapid loss of metabolic competence with suspension primary hepatocytes or subcellular fractions limits our ability to assess lower turnover compounds

• Traditional in vitro assays for metabolic clearance with suspensions of primary hepatocytes do not generally employ physiologically-relevant levels of plasma proteins that may contribute to poorer predictions

• ‘Early’ PHH cultures and NextGen in vitro liver models have shown promise in improving our ability to predict in vivo metabolism

• Additional research is needed to define metabolic ‘competence’ grounded in metabolite profiles to reflect stages of cell/tissue differentiation and development (e.g. neonate hepatocytes?)

• In silico tools are emerging, but to date require additional development to cover a broader transformation space
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