Evaluation of BSEP Inhibition and Compensatory Mechanisms in Drug Development

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Drug Induced Liver Injury (DILI)

- DILI is the leading cause of acute liver failure in the US, and a major reason for liver transplantation.¹
  - Approximately 55,000 cases/year in the US ²

- DILI is the #1 cause of regulatory actions
  - drug failure in clinical trials
  - drug withdrawal

- Herbals and dietary supplements are the second leading cause for liver injury ³

- Numerous DILI Mechanisms

- Cholestatic-DILI
  - Drug exposure disrupts bile acid homeostasis within hepatocytes
  - Accumulation of bile acids within hepatocytes lead to bile acid-induced hepatotoxicity

¹ Reuben et al. Hepatology 2010;52: 2065-2076
² Fontana. Gastroenterology 2013;314: 1818
³ Chalasani et al. Gastroenterology 2008;135:1924-1934, 1934.e1-
Historical Cholestatic DILI Hypothesis

- Normal Vectorial Flow of Bile Acids
  - Uptake (NTCP) into hepatocyte
  - Excreted (BSEP) out of hepatocyte to bile canaliculi

- BSEP inhibition results in build up of bile acids (detergents) which can “dissolve” membranes at high intracellular concentrations, leading to hepatotoxicity

- BSEP inhibition = Hepatotoxicity
  - Progress familial intrahepatic cholestasis II (PFIC II)
    - Rare genetic disorder caused by mutations in ABCB11 (BSEP)
    - Progress liver disease beginning at infancy usually ending with liver failure
In Vitro Potency of BSEP Inhibition and Cholestatic Drug Induced Liver Injury

Dawson et al., Drug Metab Dispos 40:130, 2012

False Negative
(1 non-oral drug; IC₅₀ > 1000 μM)

False Positive
(praziquantel)
(clobetasol propionate)

Weak relationship

DILI Class

Dawson et al., Drug Metab Dispos 40:130, 2012
## Predictive Power of BSEP Inhibition for Liver Injury

<table>
<thead>
<tr>
<th>Compound</th>
<th>hBSEP (µM)</th>
<th>Actual</th>
<th>Reported Hepatotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin A</td>
<td>0.5</td>
<td>FP</td>
<td>None or rare</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>0.5</td>
<td>FP</td>
<td>None or very rare</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>3</td>
<td>TP</td>
<td>1:1000</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>3</td>
<td>TP</td>
<td>1:2000</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>36</td>
<td>FN</td>
<td>Boxed warning</td>
</tr>
<tr>
<td>Imatinib</td>
<td>25</td>
<td>FP</td>
<td>Mixed</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>25</td>
<td>FP</td>
<td>1:100000</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>36</td>
<td>FN</td>
<td>1:10000</td>
</tr>
<tr>
<td>Deferasirox</td>
<td>58</td>
<td>FN</td>
<td>Boxed warning</td>
</tr>
</tbody>
</table>

### Table 1: Comparison of various assays measuring key mechanisms of toxicity endpoints associated with DILI (adapted from ref. 15)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>% Correct (positive predictive value, PPV)</th>
<th>% DILI missing (false negative rate, FNR)</th>
<th>% Accuracy (true positive + true negative)/106</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>71.9%</td>
<td>52.1%</td>
<td>69.1%</td>
</tr>
<tr>
<td>TDI</td>
<td>75.0%</td>
<td>81.3%</td>
<td>61.8%</td>
</tr>
<tr>
<td>Cytotoxicity (3T3 cells)</td>
<td>48.3%</td>
<td>70.8%</td>
<td>55.5%</td>
</tr>
<tr>
<td>Mitotox</td>
<td>71.4%</td>
<td>79.2%</td>
<td>61.8%</td>
</tr>
<tr>
<td>BSEP</td>
<td>69.2%</td>
<td>62.5%</td>
<td>65.5%</td>
</tr>
<tr>
<td>All assays</td>
<td>65.1%</td>
<td>14.6%</td>
<td>73.6%</td>
</tr>
</tbody>
</table>

- **Sensitivity:** 60%
- **Specificity:** 50%
- **Accuracy:** 22%

False Positives and False Negatives are a serious issue
Not Much Better than a Coin Toss!

Findings consistent with Dawson et al., *Drug Metab Dispos* 40:130, 2012

Proposed Threshold of 25 µM


Chan & Benet *Toxicol. Research* 2018, 7, 358-370

- Chan & Benet *Toxicol. Research* 2018, 7, 358-370
BSEP Vesicle Assay Limitations

- BSEP vesicle is an “inside-out” assay
  - Not physiological, BSEP is an internal transporter

- Vesicle Assays focus on a single transporter, and do NOT account for other uptake and/or efflux pathways.
  - False Positives
    - Low BSEP IC$_{50}$ ≤ 25 uM
    - Intracellular concentration may not reach concentrations required for inhibition
  - False Negatives
    - High BSEP IC$_{50}$ > 25 uM
    - Intracellular concentration required for inhibition achieved due to high cellular accumulation

- Lack of metabolic capacity can lead to false negatives
- Does NOT account for the Adaptive Response
Integrate Key Components for a Predictive Hepatic Model:

✓ **Uptake**
  - Sinusoidal uptake transport proteins

✓ **Efflux**
  - Biliary and/or basolateral transport proteins

✓ **Metabolism**
  - Metabolic enzymes for elimination, or generation of active/toxic metabolites

✓ **Regulation**
  - Induction of transport and metabolism

The **Intracellular Concentration** is the driving force for:
  - Hepatotoxicity
  - Efflux based interactions
  - Metabolism – induction/inhibition
A Polarized System is Critical for *In Vivo* Relevant Transporter Function

- Systems are not polarized
- Canalicular efflux transporters are internalized and **NOT** functioning
- Uptake and basolateral efflux transporters only
- Limited regulation

**B-CLEAR® Sandwich-Cultured Hepatocytes**

- Normal cell polarity re-established
- Uptake and efflux transporters functioning
- Regulatory pathways are intact and functioning
Importance of Transporter Function

- Lack of efflux transporters in plated hepatocytes generates higher intracellular concentrations, leading to hepatotoxicity

- Polarized system required to generate in vivo relevant intracellular concentrations

Each plate configuration used the same lot of Transporter Certified™ cryopreserved human hepatocytes
Adverse Outcomes Pathway: Integration of the Adaptive Response to Predict Cholestasis

Vinken M. (2013) Toxicology 312 158-165
**Predicting Cholestatic DILI**

**Historical Hypothesis:**
- BSEP inhibition results in build up of bile acids (detergents) which can “dissolve” membranes at high intracellular concentrations, leading to hepatotoxicity

**However,** bile acid disposition is tightly regulated by the Farnesoid X Receptor (FXR), and activation of FXR leads to:
  - Increased FGF19 and SHP
  - Suppression of CYP7A1
  - Induction of BSEP, MDR3, OSTα/β

Under normal circumstances, the liver has a high ability to compensate for increases in bile acid concentrations (**Adaptive Response**)
Increased Intracellular Bile Acid Concentrations - Adaptive Response

- In response to high intracellular concentrations of bile acids:
  - Decreased expression of CYP7A1
  - Increased expression of BSEP
  - Increased expression of OSTα and OSTβ
- Increase in mRNA expression of transporters linked to function
- The Net Effect of the Adaptive Response is a decrease in the intracellular concentration of bile acids

All studies in Transporter Certified™ Human Hepatocytes

CDCA ≡ chenodeoxycholic acid
BSEP Inhibition “Triggers” Adaptive Response

Exposure to Cyclosporine A (10 µM), a potent BSEP inhibitor leads to a rapid, time dependent decrease in biliary excretion of endogenous bile acids.

Inhibition of biliary excretion leads to an increase in the intracellular concentration of endogenous bile acids.

Increased intracellular concentrations of bile acids activate FXR (increased FGF19)
- This leads to suppression of CYP7A1 (bile acid synthesis), and induction of OST α/β (basolateral efflux transporter)

Jackson JP, Freeman KM, St. Claire III RL, Black CB, and Brouwer KR. Cholestatic DILI: A Function of BSEP Inhibition and FXR Antagonism. Applied In Vitro Toxicology, Vol 4, No 3, 2018
Change in mRNA Translates to Changes in Protein and Function

Impact of FXR Antagonism on the Adaptive Response

- Synergistic effect on activation of FXR in the presence of CDCA and CDCA + CsA
- Troglitazone (weak FXR antagonist) response decreased to 46.8 % of control
- DY268 (strong FXR antagonist) response decreased to 5.6 % of control
- FXR antagonism prevents the hepatocyte from responding to high intracellular concentrations of bile acids

Experimental: 24 hours exposure, Transporter Certified™ human hepatocytes in sandwich configuration (24-well) using QualGro™ media

Jackson JP, Freeman KM, St. Claire III RL, Black CB, and Brouwer KR. Cholestatic DILI: A Function of BSEP Inhibition and FXR Antagonism. Applied In Vitro Toxicology, Vol 4, No 3, 2018

CsA ≡ Cyclosporine A
CDCA ≡ Chenodeoxycholic acid
Trog ≡ Troglitazone
DY268 ≡ FXR Antagonist
The C-DILI™ Assay: Key Features

- Transporter Certified™ human hepatocytes
- 96-well plate format
- Optimized culture conditions
  - 5 days in culture: optimizes formation of bile pockets and efflux transporter function
  - QualGro™ Sensitization Media: Creates a sensitized cellular environment using lipids and bile acids
- Standard Culture Media (control)
  - Non-sensitized cells to account for direct compound toxicity
- Positive, negative and direct toxicity controls
- 24-hour incubation with test article
  - Integrates metabolism and FXR gene expression changes (Adaptive Response)
- LDH and ATP readout for toxicity

Patent Pending
C-DILI™ Assay: A Closer Look at the Data

High Toxic Potential: >200% Solvent Control

* Dunnett’s p-value ≤ 0.05
Identification of a Positive Control

• SCHH were treated for 24 hours with test article under either standard or sensitization medium conditions.

• Cytotoxicity was measured by leakage of LDH and by depletion of ATP content.

• None of the compounds significantly increased LDH secretion or depleted ATP content under standard medium conditions.

• Exposure to CsA, a BSEP inhibitor, from 1 to 25 µM (~C\text{max} to 31X C\text{max}) had no effect.

• However under sensitization conditions, Troglitazone (100 µM; 16X C\text{max}) was the only compound that significantly increased LDH secretion.

• Assay can differentiate the cholestatic hepatotoxicity of troglitazone from rosiglitazone, and pioglitazone.
Integration of Multiple Mechanisms to Produce Hepatotoxicity

- Troglitazone and its sulfate metabolite inhibit BSEP
- Troglitazone is a weak FXR antagonist
- Troglitazone sulfate is also an inhibitor of the basolateral efflux transporters OSTα/β *

- Toxicity is only observed when compounds impact multiple pathways
  - Inhibition of BSEP and/or basolateral efflux
  - FXR gene regulation (e.g. FXR antagonists)

At high concentrations cyclosporine A is toxic
Increasing Bile Acid concentration leads to hepatotoxicity
Cyclosporine A, a potent BSEP inhibitor (IC$_{50}$ ~ 0.5µM) does NOT show toxicity greater than DMSO control
CCAAT/enhancer-binding protein homologous protein (CHOP) is a key marker of ER stress and early initiator of cell death.

- ER stress initiates bile acid induced programmed cell death.
- CsA (BSEP inhibition) and DY268 (FXR antagonist) were negative. Each only has one of the required characteristics for bile-induced hepatotoxicity.

**Troglitazone** has BSEP inhibition, FXR antagonism, and OSTα/β inhibition. Concomitant increases of CHOP mRNA and LDH leakage only in hepatocytes treated with Troglitazone under sensitization conditions.
Cholestatic Hepatotoxicity Positives Assessed for FXR Antagonism

Bile-induced Hepatotoxicity (C-DILI)

- Compounds that inhibit bile acid efflux and antagonize FXR or block basolateral efflux
- Ketoconazole, deferasirox, troglitazone **reduce the effectiveness** of the FXR-dependent compensatory mechanism
**Cholestatic DILI: Hepatocellular Injury**  
Need to integrate multiple mechanisms

**Initiating Insult**
- BSEP Inhibition

**Secondary Insult**
- FXR Antagonism and/or
- Basolateral Efflux Inhibition

Compounds can Increase the Intracellular Concentration of Bile Acids through:
- BSEP Inhibition **plus**
- Basolateral Efflux Inhibition (MRP3/4 and/or OSTα/β) and/or
- FXR Antagonism

Jackson JP, Freeman KM, St. Claire III RL, Black CB, and Brouwer KR.  
Cholestatic DILI: A Function of BSEP Inhibition and FXR Antagonism.  
Applied In Vitro Toxicology, Vol 4, No 3, 2018
Changing Opinions: International Transporter Consortium Perspective

BSEP inhibition concern identified in vesicle assay

Transfected Cell Systems: specific transporter overexpressing system

Assess:
• Screen for parent and metabolite(s) inhibition of other bile acid transporters (NTCP, OATPs, MRP3, MRP4, MRP2, OSTα/β)
• Correlation of transporter inhibition with drug exposure

Predict:
• NTCP/OATP inhibition may serve as a protective mechanism
• MRP2/3/4 and/or OSTα/β inhibition may further increase hepatic bile acid concentrations

Whole Cell System: Sandwich-Cultured Human Hepatocytes

Assess:
• Integration of multiple mechanisms
• Inhibition of $CL_{uptake}$, $CL_{biliary}$, and/or $CL_{basolateral efflux}$
• Nuclear receptor regulation of bile acid transport and synthesis

Measure:
• Intracellular parent and metabolite concentrations
• Alterations in intracellular bile acid concentrations
• Concentration-dependent hepatotoxicity

Quantitative Systems Toxicology Modeling

Develop mechanistic models:
• Normal and disease states
• Integrate multiple bile acid pathways (e.g., transport, metabolism)
• Evaluate competitive and non-competitive processes
• Integrate multiple mechanisms (e.g., mitochondrial toxicity, reactive metabolites)

Predict:
• Incidence and time course of hepatotoxicity in humans and preclinical species
• Range of hepatotoxic responses in various populations
• Patient susceptibility factors for DILI

Importance of the Adaptive Response to BSEP Inhibition

Inclusion of the **adaptive response** improves DILI prediction accuracy

- BSEP inhibition “triggers” the adaptive response
- A secondary insult required to cause cholestatic DILI such as:
  - Basolateral Efflux Inhibition (MRP3/4 and/or OSTα/β) and/or
  - FXR Antagonism

It is the **NET effect** of all these processes on bile acid disposition (adaptive response) that determine the cholestatic drug induced liver injury potential of a compound.
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