Metabolically-modified liver models to examine the role of mitochondrial dysfunction in drug-induced liver injury and their relevance to man

Dr Amy Chadwick

The University of Liverpool, UK
Interests in drug-induced mitochondrial toxicity

1) Assess potential for inducing ADR (MIP-DILI consortium)
2) Investigate factors influencing susceptibility
3) Evaluate potential clinical biomarkers of dysfunction
Mechanism–based Integrated Systems for the Prediction of Drug-Induced Liver Injury (MIP DILI)

**IMI 3rd call topic**

“Improved early prediction of drug induced Liver Injury (DILI) in man”

**RESEARCH ACADEMICS GROUP**

**EFPIA MEMBERS**

**SMEs**

26 Partners
**PRIMARY GOAL:** To identify and validate an improved panel of *in vitro* “best practice assays” for predicting DILI in the human population

**SUPPORTIVE GOALS:**

- To explore the relationship between *in vitro* and *in vivo* assay signals
- To develop and evaluate novel modelling approaches
- To enhance academia -pharmaceutical - regulatory understanding

*In vitro test systems*
MIP DILI: Project Strategy

- **in vitro** test systems
  - Current and emerging cell systems
  - Refined cell systems
  - Multi-cell systems

- compound training sets
  - Mechanism specific hepatotoxins
  - Pathway selective compounds
  - Diagnostic test sets

- **in vivo** models
  - Current and emerging in vivo models
  - Refined in vivo models
  - Humanized in vivo models

Novel mechanism-based systems for DILI prediction
DILI and Mitochondria Dysfunction: a myth or the truth?

Withdrawn/Discontined Post-marketing:
Troglitazone, Nefazodone

Drug Attrition Pre-Marketing:
Fialuridine, Panadiplon

Drugs with Blackbox Warning:
Amiodarone, Tolcapone,
NRTI’s, Valproic Acid

Evidence for mitochondrial liability but:
- not proven connection in man
- Mechanism not clear
DILI and Mitochondria Dysfunction: Variability in mitochondrial toxicity

MITOCHONDRIAL TARGETS

- mtDNA
- β-oxidation
- Electron transport chain
- Tricarboxylic acid cycle
- Mitochondrial pore

INDIVIDUAL VARIATION

- mtDNA genetic polymorphisms
- Metabolic capacity (mutations in mtDNA)

1. Drugs containing mitochondrial liabilities induce unpredictable ADRs due to variation in mtDNA.
2. Models must reproduce the variation in respiratory capacity observed clinically in order to confer susceptibility to drugs that are mitotoxins.

Models adaptable to animal and human samples: e.g. fresh hepatocytes

Patient variability in susceptibility to mitochondrial toxins

Difficult to investigate preclinically in “healthy rodents” or cell models with abnormal respiration

Contemporary Issues in Toxicology
Mitochondrial abnormalities—A link to idiosyncratic drug hepatotoxicity?

Urs A. Boelsterli a,b,*, Priscilla L.K. Lim a

HepG2 Glucose/Galactose Model

more susceptible to mitotoxicants

Immortal Cell Line

Galactose Cell Line

ATP = OXPHOS only

Circumventing the Crabtree Effect: Replacing Media Glucose with Galactose Increases Susceptibility of HepG2 Cells to Mitochondrial Toxicants

Lisa D. Marroquin,† James Hynes,‡ James A. Dykens,§ Joseph D. Jamieson,∥ and Yvonne Will*†‡

TOXICOLOGICAL SCIENCES 97(2), 539–547 (2007)

Cells converted over 8 passages
HepG2 Glucose/Galactose Model in Industry

Questionnaire to pharma partners on use of HepG2 for preclinical screens

8/8 used HepG2 screens

6/8 routinely use for mitotox testing

All 6/8 use galactose media for mitotox testing

Information used to rank compounds, inform structure activity relationships and/or inform drug design.

HepG2

- Human Heptocellular Carcinoma
- Widely used for screening in industry
- Absence of xenobiotic metabolism (CYP P450s)
**HepG2 Glucose/Galactose Model**

*more susceptible to mitotoxicants*

**Immortal Cell Line**
- **Glycolysis**
- ATP

**Galactose Cell Line**
- **Glycolysis**
- ATP

Glucose free culture + *galactose, glutamine*

ATP = OXPHOS only

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**Rotenone (24 h, MTT)**

```
<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Cell Viability (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001</td>
<td>120</td>
</tr>
<tr>
<td>0.001</td>
<td>100</td>
</tr>
<tr>
<td>0.01</td>
<td>80</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>
```

- ○ HepG2
- ● HepG2 + Galactose

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*TOXICOLOGICAL SCIENCES 97(2), 539–547 (2007)*

*Cells converted over 8 passages*
Modified Metabolic Switch

ADVANTAGES

1. Compare the effects of metabolic modification on drug sensitivity in cells originating from the same source.

2. Extends application to other cell models such as fresh primary hepatocytes.

3. Short exposure time allows examination of the role of mitochondrial dysfunction in the absence of cell death.
MIP DILI Training Compounds

DIRECT MITOCOCHONDRIAL TARGETS

- Perhexiline
- Fialuridine

- mtDNA
- β-oxidation
- Tricarboxylic acid cycle
- Electron transport chain
- Mitochondrial pore

Compounds:
- Amiodarone
- Perhexiline
- Nefazodone
- Buspirone
- Troglitazone
- Pioglitazone
- Diclofenac
- Metformin
- Paracetamol
- Tolcapone
- Rotenone, CCCP, Antimycin A
- Diclofenac
- Troglitazone
- Paracetamol
- Bosentan
- Entacapone
- Digitonin

MIP-DILI
Results: Positive Controls (2 h)

Window of mitochondrial dysfunction before cell death
Results: MIP-DILI cmpds (2 h)

Nefazodone

Troglitazone

Amiodarone

Tolcapone

ATP-HepG2-Glucose
ATP-HepG2-Galactose
Cytotoxicity-HepG2-Glucose
Cytotoxicity-HepG2-Galactose

Concentration (µM)

Concentration (µM)

ATP (% of vehicle Control)

Cytotoxicity (% of vehicle control)

ATP (% of vehicle Control)

Cytotoxicity (% of vehicle control)

ATP (% of vehicle Control)

Cytotoxicity (% of vehicle control)

ATP (% of vehicle Control)

Cytotoxicity (% of vehicle control)
## Results: MIP-DILI compounds (2 h)

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$-ATP</th>
<th>Ratio EC$_{50}$: ATPglut /ATPgal</th>
<th>P value:</th>
<th>EC$_{150}$-CT</th>
<th>Ratio EC$<em>{50}$: CT$</em>{a}$gal / ATPgal</th>
<th>P value:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose (Glu)</td>
<td>Galactose (Gal)</td>
<td></td>
<td>Glucose (Glu)</td>
<td>Galactose (Gal)</td>
<td></td>
</tr>
<tr>
<td>Amiodarone</td>
<td>&gt;300</td>
<td>157.5</td>
<td>&gt;2</td>
<td>&gt;300</td>
<td>195.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>38.8</td>
<td>16.6</td>
<td>2.3</td>
<td>75.8</td>
<td>142.2</td>
<td>8.5</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>81.3</td>
<td>28.4</td>
<td>2.9</td>
<td>&gt;300</td>
<td>83.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>&gt;300</td>
<td>53.2</td>
<td>5.6</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;5.6</td>
</tr>
<tr>
<td>Buspirone</td>
<td>&gt;900</td>
<td>337</td>
<td>&gt;2.7</td>
<td>&gt;900</td>
<td>&gt;900</td>
<td>&gt;2.6</td>
</tr>
</tbody>
</table>

Positive for mitochondrial toxicity
All previously reported to induce mitochondrial dysfunction via ETC
Results: Extended Timecourse & Concentration Curve

Fialuridine and ximelagatran remain negative

HPLC-MS/MS showed no significant metabolism of APAP or ximelagatran
Ranking of Mitotoxins: Matches Clinical Toxicity Potential

Troglitazone vs Pioglitazone
(2 h, 28 μM vs 4 h, 1 mM)

Nefazodone vs Buspirone
(2 h, 17 μM vs 2 h, 337 μM)

Entacapone mitotoxicity only at 4 h

<table>
<thead>
<tr>
<th>Compound</th>
<th>DILI</th>
<th>Hepatotoxic</th>
<th>Non-hepatotoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolcapone</td>
<td>CDSS Mito Screen Positive Result</td>
<td>2 h</td>
<td>IC_{50} galA TP 53 μM</td>
</tr>
<tr>
<td>Entacapone</td>
<td></td>
<td></td>
<td>4 h, 1 mM</td>
</tr>
</tbody>
</table>

Literature

**Strong**: uncoupler, interacts with ETC complex proteins, FAO, bile acid synthesis. Forms MPT pores, decreases MMP

**Weak**: mild uncoupling effect
## MIP-DILI Mitotox Analysis vs Literature

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mitochondrial Liability Literature</th>
<th>MIP-DILI Mitotox Analysis</th>
<th>Hepatotoxic</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>Yes – Inhibits ATP synthase, MPTP opener, mitochondrial ROS,</td>
<td>Positive Extended</td>
<td>Yes</td>
<td>Parmar et al, 1995, Kon et al., 2004</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Yes – OXPHOS uncoupler, inhibits fatty acid oxidation via CPT1 inhibition</td>
<td>Positive Standard</td>
<td>Yes</td>
<td>Fromenty et al., 1990 Kennedy et al., 2006</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>Yes - complex I &gt; complex IV inhibition of ETC in HepG2 and isolated rat liver</td>
<td>Positive Standard</td>
<td>Yes</td>
<td>Dykens et al., 2008</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>Yes - OXPHOS uncoupler, interacts with ETC complex proteins, FAO, bile acid synthesis. Forms MPT pores, decreases MMP</td>
<td>Positive Standard</td>
<td>Yes</td>
<td>Korlipara, Cooper and Schapira, 2004</td>
</tr>
<tr>
<td>Entacapone</td>
<td>Yes – mild OXPHOS uncoupler</td>
<td>Positive Extended</td>
<td>No</td>
<td>Korlipara et al., 2004</td>
</tr>
<tr>
<td>Bosentan</td>
<td>No</td>
<td>Negative</td>
<td>Yes</td>
<td>Clinical Pharmacology &amp; Therapeutics (2001) 69, 223–231</td>
</tr>
<tr>
<td>Buspirone</td>
<td>Yes - complex I inhibition of ETC in HepG2 and isolated rat liver</td>
<td>Positive Standard</td>
<td>Yes</td>
<td>Dykens et al., 2008</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>Yes – MPTP opener, mild OXPHOS uncoupler, inhibits ATP synthase and adenine nucleoside translocase</td>
<td>Positive Extended</td>
<td>Yes</td>
<td>Moreno-Sanchez et al., 1999</td>
</tr>
<tr>
<td>Metformin</td>
<td>Yes – complex I inhibitor</td>
<td>Positive Extended</td>
<td>No</td>
<td>Carvalho et al., 2008</td>
</tr>
<tr>
<td>Ximelagatran</td>
<td>No</td>
<td>Negative</td>
<td>Yes</td>
<td>Kenne et al., 2008</td>
</tr>
<tr>
<td>Fialuridine</td>
<td>Yes – impairs mtDNA replication,</td>
<td>Negative (chronic toxicity)</td>
<td>Yes</td>
<td>Lewis et al., 1996</td>
</tr>
<tr>
<td>Perhexiline</td>
<td>Yes – Inhibits carnitine uptake via CPT1, inhibits fatty acid oxidation</td>
<td>Negative (mechanistic factors)</td>
<td>Yes</td>
<td>Kennedy et al., 2006</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>Yes – reported inhibitor of complex I, II, III, IV, V inhibitor, MPTP opener, OXPHOS uncoupler</td>
<td>Positive Standard</td>
<td>Yes</td>
<td>Nadanaciva, 2008 Scatena et al., 2004</td>
</tr>
</tbody>
</table>
ATP/cytotoxicity assessment reflects the effects of the compounds upon cellular respiration.

Cells plated on collagen (overnight)
Media changed (glucose/galactose, 1 h)
Drugs injected in situ (ATP EC50\textsubscript{gal})
Basal OCR & ECAR (2 h)

**Cellular Bioenergetic Phenotyping**

**Rotenone**

**CCCP**
ATP/cytotoxicity assessment reflects the effects of the compounds upon cellular respiration.

Distinct bioenergetic profiles may be associated with the target of mitochondrial dysfunction and may be used to direct further mechanistic studies.
A glance backstage: Mitochondrial Imaging

- Glucose media + serum
- Approx ATP$_{50gal}$ conc (2 h)
- Staining HSP 60

![Control](image1)
![CCCP](image2)
![Troglitazone](image3)
![Nefazodone](image4)
![Amiodarone](image5)
Does the HepG2 Model represent Fresh Human Hepatocytes?

**Rotenone (4 h) ATP**  
**Glucose vs galactose media (MIP-DILI model)**

Comparable response to rotenone between HepG2_gal and FHH  
FHH cells cannot be used to classify toxicants as mitotoxicants
Mito-tox assay on fresh human hepatocytes (FHH). The cells were cultured in glucose or galactose media for four hours, then exposed to rotenone (0-100 µM) for 2 hours. ATP and cytotoxicity assay were performed.

The graph shows the concentration of rotenone that reduced the ATP level in glucose or galactose media by 50% (EC$_{50}$-ATP) or increased the cytotoxicity by 50% (EC$_{150}$-CT) in each media.

Differences in cytotoxicity between donors suggests variation in how an individual can respond to dysfunction.
Conclusions

1. HepG2 cells acutely deprived of glucose are a suitable model for the identification of mitochondrial toxicity when used alongside HepG2 cells in the presence of glucose.

2. The dual assessment of ATP content alongside cytotoxicity provides an enhanced mechanistic understanding of the causes of toxicity.

3. The utility of this assay to identify compounds which induce dysfunction of the ETC directly has been proven, with zero to low levels of false positive results.

4. The results from the screen are supported by other functional and morphological data.

5. Limitations of the screen include detecting alternative mechanisms of mitochondrial dysfunction or mitotoxicity induced via reactive metabolites.

6. The model developed is able to give information on the potency of a series of compounds which may be indicative of the propensity to cause hepatotoxicity.
Suggested Recommendations to EFPIA

This screen should be used only to identify potential mitotoxins

There is no link to peak plasma concentrations

Sensitive first-tier screening

HepG2 glu/gal screen (parent compound & reactive metabolites)

- Identify potential mitotoxins
- Rank compounds
- Starting point for mechanistic studies
- Inform preclinical testing strategy
Drug

Cell function
- Specialised cell systems
  - Transporter inhibition
- Cholestasis

Pharmacological liability
- Hepatocyte suspension (1-4h)
  - Accumulation
- Bioactivation

Phenotype

Cell health
- HepG2
  - Cytotoxicity
  - Mitotoxicity

Cell function

 tier 1

Multi-cell culture
Long term culture
2D, 3D Spheroids

 tier 2

Patient dependent factors
- Complex systems
  - Viral infection
  - HLA restriction
  - T cells

 tier 3

MITOTOX specific analysis

Seahorse Analysis
Transcriptome
Proteome
Mitotoxicity
Imaging
Modelling
Bridging Biomarkers
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