Innovative in vitro models of toxicology assessments

Chris Goldring
University of Liverpool

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Drug-Induced Liver Injury

Post-marketing withdrawal of 462 medicinal products because of adverse drug reactions: a systematic review of the world literature

Igho J. Onakpoya*, Carl J. Heneghan and Jeffrey K. Aronson

BMC Med. 2016 Feb 4;14:10

From 1953-2013 462 drugs were withdrawn post-marketing due to ADRs.

- Hepatotoxicity (81 cases; 18%)
- Immune-related reactions (79 cases; 17%)
- Cardiotoxicity (63 cases; 14%)
Drug-Induced Liver Injury

- Dose-dependent
- Species selective
- Selective individuals
- Idiosyncratic
Chemical Insults and DILI

1. Mitochondrial impairment
2. Inhibition of biliary efflux
3. Lysosomal impairment
4. Reactive metabolites/Covalent Binding
   - Chemical stress
   - Immune activation
5. Inflammation Immune System
   - Innate
   - Adaptive
Chemical Insults and DILI

Diverse Clinical Presentations of DILI
- Acute fatty liver with lactic acidosis
- Acute hepatic necrosis
- Acute liver failure
- Acute viral hepatitis-like liver injury
- Autoimmune-like hepatitis
- Bland cholestasis
- Cholestatic hepatitis
- Cirrhosis
- Immuno-allergic hepatitis
- Nodular regeneration
- Nonalcoholic fatty liver
- Sinusoidal obstruction syndrome
- Vanishing bile duct syndrome

1. Mitochondrial impairment
2. Inhibition of biliary efflux
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### Genetic Restriction and Drug Hypersensitivity: Discovery of HLA Allele Associations

- **Discovery of associations** between HLA alleles and drug hypersensitivity represents an important advance.
- **Screening for HLA alleles** during clinical practice effectively prevents reactions:
  - Abacavir
  - Carbamazepine
- **This is not the case for DILI**
- **So we need biomarkers and models**

#### Pharmacogenetics and Clinical Syndromes

<table>
<thead>
<tr>
<th>Drug</th>
<th>HLA Allele</th>
<th>OR</th>
</tr>
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<tbody>
<tr>
<td><strong>Abacavir</strong></td>
<td>HLA-B*5701</td>
<td>132</td>
</tr>
<tr>
<td><strong>Flucloxacin</strong></td>
<td>HLA-B*5701</td>
<td>72</td>
</tr>
<tr>
<td><strong>Carbamazepine</strong></td>
<td>HLA-B*1502</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Carbamazepine</strong></td>
<td>HLA-A*3101</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(Japanese)</td>
<td></td>
</tr>
<tr>
<td><strong>Carbamazepine</strong></td>
<td>HLA-A*3101</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(Caucasians)</td>
<td></td>
</tr>
<tr>
<td><strong>Lumiracoxib</strong></td>
<td>HLA-DRB1*1501</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>HLA-DQA1*0102</td>
<td></td>
</tr>
<tr>
<td><strong>Ximelagatran</strong></td>
<td>HLA-DRB1*0701</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>HLA-BQA1*0201</td>
<td>9.0</td>
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<tr>
<td><strong>Lapatinib</strong></td>
<td>HLA-DRB1*1501</td>
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Mallal, 2008; Kindmark et al., 2008; Daly et al., 2009; Chung et al., 2004; McCormack et al., 2011; Singer et al., 2010; Spraggs et al., 2011
MIP-DILI Roadmap - Stratification of *In Vitro* systems

**Tier 1**
- Single cell 2D systems
  - PHH
  - Cell-lines
  - Reporter-signalling

**Tier 2**
- Multi-dimensional 3D systems
  - Multi-cell culture
  - Spheroids
  - Long term culture

**Tier 3**
- Complex systems
  - Patient-dependent factors
    - Viral infection
    - HLA restriction
    - T cells

- **Physiology**
- **Pharmacology**
- **Toxicology**
MIP-DILI Roadmap - Stratification of *In Vitro* systems

**Tier 1**

**Physiology**
- Expression phenotype
- Transcriptome
- Proteome
- Cell Signalling
- Albumin secretion
- Biomarker secretion

**Physiology**
- Function

**Pharmacology**
- Drug Accumulation
- Drug Bioactivation/Covalent Binding
- Drug Detoxication

**Toxicology**
- Cytotoxicity
- Transporter inhibition
- Cholestasis
- Lysosomal impairment
- Mitotoxicity
- GSH depletion
- ER stress
- Oxidative stress

**Single cell 2D systems**

**Tier 2**

**Multi-dimensional 3D systems**
- Multi-cell culture
- Spheroids
- Long term culture

**Tier 3**

**Complex systems**
- Viral infection
- HLA restriction
- T cells

**Patient-dependent factors**

**Databases**
- Chemoinformatics

**Bioanalysis**
- Transcriptomics

**Proteomics**
- Metabolic profiles
- Drug distribution
- Biomarkers

**Functional measurements**
- Drug metabolizing enzyme activity
- Transporter activity
- High content analysis
- Innate Signalling
- Adaptive response

**Mathematical modelling**
Selection of Model Systems

Importance of the physiological and pharmacological phenotype for the application of a toxicological test

- Is a particular test system fit for purpose?
- What purpose is it fit for?
Importance of the physiological and pharmacological phenotype for the application of a toxicological test

**TIER ONE**

Tier one cell types

- Primary Hepatocytes
  - Fresh
  - Cryo-preserved
- Liver-derived cell lines
  - HepG2
  - HepaRG
  - UpCytes

iTRAQ proteomic comparison
Biological Replicate | No. of proteins identified | Proteins quantified
--- | --- | ---
1 | 4335 | 3197
2 | 4887 | 4397
3 | 4891 | 3794
Total (common to all replicates) | 2726

Comparative Proteomic Characterization of 4 Human Liver-Derived Single Cell Culture Models Reveals Significant Variation in the Capacity for Drug Disposition, Bioactivation, and Detoxication

Rowena L. C. Sison-Young, Dimitra Mitsa, Rosalind E. Jenkins, David Mottram, Eliane Alexandre, Lysiane Richter, Hélène Aerts, Richard J. Weaver, Robert P. Jones, Esther Johann, Philip G. Hewitt, Magnus Ingelman-Sundberg, Christopher E. F. Goldring, Neil R. Kitteringham, and B. Kevin Park
HLCs Proteomic Phenotype

Phase II drug metabolising enzymes

Cytochrome P450s

Transporters
<table>
<thead>
<tr>
<th>Compound</th>
<th>Mitochondrial Liability Literature</th>
<th>MIP-DILI Mitotox Analysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>Yes – Inhibits ATP synthase, MPTP opener, mitochondrial ROS,</td>
<td>Positive</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</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</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</td>
<td>Korlipara, Cooper and Schapira, 2004</td>
</tr>
<tr>
<td>Entacapone</td>
<td>Yes – mild OXPHOS uncoupler</td>
<td>Negative</td>
<td>Korlipara et al., 2004</td>
</tr>
<tr>
<td>Bosentan</td>
<td>No</td>
<td>Negative</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</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</td>
<td>Moreno-Sanchez et al., 1999</td>
</tr>
<tr>
<td>Metformin</td>
<td>Yes – complex I inhibitor</td>
<td>Positive</td>
<td>Carvalho et al., 2008</td>
</tr>
<tr>
<td>Ximelagatran</td>
<td>No</td>
<td>Negative</td>
<td>Kenne et al., 2008</td>
</tr>
<tr>
<td>Fialuridine</td>
<td>Yes – impairs mtDNA replication,</td>
<td>Negative (chronic toxicity)</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>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</td>
<td>Nandanaciva, 2008, Scatena et al., 2004</td>
</tr>
</tbody>
</table>
Fialuridine – *fatal clinical trial*

- Developed as an antiviral for HIV – later considered as a treatment for hepatitis B
- Preclinical testing in mice, rats, dogs and monkeys

### Clinical Testing

<table>
<thead>
<tr>
<th>Patients</th>
<th>Duration</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+/CMV+ (12)</td>
<td>35 d</td>
<td>Duration prolonged to sustain antiviral (HBV+)</td>
</tr>
<tr>
<td>HIV+/HBV+ (43)</td>
<td>14 d</td>
<td>No signs of toxicity</td>
</tr>
<tr>
<td>HBV+ (24)</td>
<td>28 d</td>
<td></td>
</tr>
<tr>
<td>HBV+ (15)</td>
<td>6 mth</td>
<td>6 mth planned, terminated at wk 13</td>
</tr>
</tbody>
</table>

**Sudden Hepatotoxicity & Pancreatitis**
- 5 patients died (ALF)
- 2 survived after emergency liver transplant
- 3 recovered
- 3 showed no adverse effects (lower doses)

### Clinical Features of Toxicity
- Delayed (from week 13)
- Lactic Acidosis
- Micro and macro vesicular hepatic steatosis
- Abnormal mitochondria

*Indicative of mitochondrial dysfunction*

McKenzie et al, 1995 (333)
Fialuridine *in vitro* requirements

**Fialuridine**

**KEY FEATURES OF MECHANISM OF TOXICITY**

- Human specific (via hENT mitochondrial localisation)
- Bioactivation to triphosphate (mitochondrial thymidine kinases)
- Delayed onset (wk 13 of clinical trial)
- Sudden and rapid acceleration of toxicity (threshold effect)
- Targets mitochondrial DNA replication
- Lactic acidosis and steatosis in humans
Fialuridine *in vitro* models: HepG2 vs HepaRG

<table>
<thead>
<tr>
<th>Cmax</th>
</tr>
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<tr>
<td>Healthy volunteers (5 mg dose)</td>
</tr>
<tr>
<td>Patients with DILI (0.1 – 0.25 mg/kg/day)</td>
</tr>
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</table>

**HepG2:** Max time course 7 days

<table>
<thead>
<tr>
<th>Fialuridine Concentration (µM)</th>
<th>Cellular ATP and retained LDH level relative to vehicle control (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>LDH-Glucose</td>
</tr>
<tr>
<td>10</td>
<td>ATP-Glucose</td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
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Cytotoxicity in HepG2 at high concentrations not related to the clinical mitochondrial mechanism of action.

**HepaRG:** Max time course 5 weeks

<table>
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<th>Fialuridine Concentration (µM)</th>
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Extended dosing in HepaRG allows mitochondrial toxicity to develop.

Negative mitotoxin

Positive mitotoxin
Evidence for Mitoxicological Mechanism seen in Man

- **FIAU**
  - Mitochondrial uptake
  - **Phosphorylation**
    - Incorporation/inhibition of Pol γ
    - ↓ mtDNA replication
    - ↓ mito-encoded respiratory complexes
    - ↓ OXPHOS and ↑ Glycolysis
    - ↑ Lactate
    - ↓ ATP
    - Cell stress
    - Cell death

- **BIOACTIVATION**

Intracellular presence of FIAU-MP, -DP and –TP (LC-MS)
Evidence for Mitoxicological Mechanism seen in Man

- FIAU
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  - Incorporation/inhibition of Pol γ
  - mtDNA replication
  - ↓ mtDNA replication
  - ↓ mito-encoded respiratory complexes
  - ↓ OXPHOS and ↑ Glycolysis
  - ↑ Lactate
  - ↓ ATP
  - Cell stress
  - Cell death

Graph: DECREASED MtDNA LEVELS
- Quantified using PCR
- (mtDNA and nuDNA quantified)
Evidence for Mitoxicological Mechanism seen in Man

- **FIAU**
  - Mitochondrial uptake
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- **Decreased oxidative phosphorylation**
  (using Seahorse Technology to measure oxygen consumption)

- **ATP-linked respiration**
  Indicates the oxygen consumption which is linked to ATP-production by OXPHOS

- **Bar graph**
  - OCR (pmol/min/mg protein)
  - Fialuridine (µM): 0.00, 3.70, 11.10, 33.30, 100.00
  - Colors: week 2 (green), week 4 (red)
Evidence for Mitoxicological Mechanism seen in Man

FIAU

Mitochondrial uptake

Phosphorylation

Incorporation/inhibition of Pol γ

↓ mtDNA replication

↓ mito-encoded respiratory complexes

↓ OXPHOS and ↑ Glycolysis

↑ Lactate

↓ ATP

Cell stress

Cell death

Decreased cellular ATP content followed by cell death
(11 µM over 4 weeks)
Importance of the physiological and pharmacological phenotype for the application of a toxicological test

Fialuridine

<table>
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<tr>
<th>Protein</th>
<th>HepG2 %</th>
<th>HepaRG %</th>
<th>Hepatocytes %</th>
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<tbody>
<tr>
<td>Equilibrative nucleoside transporter</td>
<td>ENT1</td>
<td>174.5</td>
<td>63.3</td>
</tr>
<tr>
<td>Thymidine kinase (cytosolic)</td>
<td>TK1</td>
<td>726.8</td>
<td>286.1</td>
</tr>
<tr>
<td>Thymidine kinase (mitochondrial)</td>
<td>TK2</td>
<td>51.5</td>
<td>73.9</td>
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**Equilibrative nucleoside transporter**

- **ENT1**
  - Primary Hepatocytes %: 100

**Thymidine kinase**

- **TK1**
  - HepG2 %: 726.8
  - HepaRG %: 286.1
- **TK2**
  - HepG2 %: 51.5
  - HepaRG %: 73.9

**Thymidylate kinase**

- **TMPK**
  - HepG2 %: 177.6
  - HepaRG %: 120.0
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Importance of the physiological and pharmacological phenotype for the application of a toxicological test

Protein | HepG2 | HepaRG | Hepatocytes |
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Equilibrative nucleoside transporter | ENT1 | 174.5 | 63.3 | 100
Thymidine kinase (cytosolic) | TK1 | 726.8 | 286.1 | 100
Thymidine kinase (mitochondrial) | TK2 | 51.5 | 73.9 | 100
Thymidylate kinase | TMPK | 177.6 | 120.0 | 100
Whole proteome: 2D, spheroid, fresh primary hepatocytes and liver from several human donors

*Data expressed relative to freshly-isolated hepatocytes from corresponding donor*
Cross-centre assessment of 3D spheroids

Compounds negative in a HCA analyses (n=35) were evaluated using the 3D spheroid system and overall 65% of compounds missed in the HCA screening were detected in the 3D spheroids as being hepatotoxic.
The application of 3D spheroids would be more focused towards lower compound throughput and/or mechanistic studies.
Regeneration and homeostasis of the liver mass: Human In vitro

Generation of long-lived human liver-like organoids from biliary ductal epithelial cells

- Evidence for a role for these cells in human liver regeneration at least in vitro
- Alternative route for generation of liver models (ie use human liver tissue bipotential stem cells)
Conclusions

- We can develop a battery test system based on current science which is fit for purpose - refinement and benchmarking.

- The implementation of novel model systems with respect to industrial application is being conducted - EFPIA workshops.

- Multidimensional \textit{in vitro} systems, which have a relevant physiological and pharmacological phenotype and are therefore fit for toxicological application(s) are being progressed - definitive qualitative and quantitative mass spectrometry

- Current status of novel model systems for idiosyncratic DILI with respect to human relevance - aspirational
## Acknowledgements

<table>
<thead>
<tr>
<th>Centre for Drug Safety Science</th>
<th>Karolinska Institute</th>
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<tbody>
<tr>
<td>• Kevin Park</td>
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<td>• Mark Bayliss</td>
<td>• Catherine Bell</td>
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<td>• Sabrina Moro</td>
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<td>• Neil French</td>
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<td>• Amy Mercer</td>
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|                                               | Servier                                      |
|                                               | • Richard Weaver                             |

|                                               | Riken Bioresource Centre                    |
|                                               | • Takao Iwawaki                              |

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