SYSTEMS–BASED APPROACH FOR PREDICTION OF DRUG-INDUCED LIVER INJURY (DILI)
Points to cover

- Overview of Heptox platform
  - Integration of *in vitro* and *in silico* approach
- Validation set of compounds
- Simulations strategy
- *Case studies*
  - TAk875 is predicted to be associated with necrotic and cholestatic potential
  - Heptox differentiates the safe compound from unsafe in a paired set of drugs e.g. Troglitazone and Pioglitazone
  - SFN priming protects system from oxidative stress
  - Prediction of cholestatic mechanism of BI201335
How the Heptox platform was created

- Build a comprehensive model of **liver homeostasis** (normal or steady state)
- Treat disease or toxicity as a case of drug/gene/environment induced perturbations
- Create assays that measure effect of drug/metabolite on the pathways
- To test a drug
  - Measure the set of assays in presence of drug/metabolite *in vitro*
  - Feed the assay results into an *in silico* model and perform simulations
What is the Virtual Liver?

• A dynamic systems model of normal liver physiology
  ▪ Built using a system of non-linear differential equations

• Allows one to assess the impact of various drugs, perturbations and insults on the liver

• Can represent the evolution from “normal” to “disease”

• Is used in conjunction with a panel of assays that link liver biology to molecule chemistry
What is it Not?

• It is **not** a database of interactions
  ▪ Virtual liver consists of processes, pathways, etc that are quantitatively linked
  ▪ This allows one to run simulations, make predictions and develop insights

• It is **not** a QSAR Model
  ▪ The Virtual Liver is a model of biology
  ▪ It is not “trained” using existing drugs or chemicals
  ▪ Hence it is not limited by a chemical space
Integration of *in vitro* and *in silico* method to predict *in vivo* behavior
DILI prediction workflow: Integrated *in vitro-in silico* approach

*U.S. Patent No. 8,645,075: A method for predicting organ toxicity and a system thereof*
Simulated model outputs are equivalent to the clinically observed DILI endpoints

<table>
<thead>
<tr>
<th>Model outputs</th>
<th>Clinically observed outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP concentration</td>
<td>Necrosis</td>
</tr>
<tr>
<td>Redox state (GSH/GSSG)</td>
<td>Oxidative stress/necrosis</td>
</tr>
<tr>
<td>Triglyceride concentration</td>
<td>Steatosis</td>
</tr>
<tr>
<td>Bile salt concentration in plasma</td>
<td>Cholestasis</td>
</tr>
</tbody>
</table>

Simulated model outputs include:

- ATP concentration
- Redox state (GSH/GSSG)
- Triglyceride concentration
- Bile salt concentration in plasma

The graphs illustrate the change in these parameters over time for different exposures and concentrations.
Virtual Liver Network

- Fatty acid transporter
- Hepatic uptake of FFA
- Diacyl glycerol synthesis
- Desaturation of fatty acid
- Mitochondrial beta-oxidation
- Fatty acid synthase
- De novo fatty acid synthesis
- Glutathione reductase
- ROS generation
- VLDL assembly
- Triglyceride synthesis
- Microsomal triglyceride transfer protein
- Carnitine palmitoyl transferase
- Gamma GCS
- Lipoprotein turnover in plasma
- Cholesterol turnover in plasma
- Phospholipid synthesis
- Tricarboxylic acid cycle
- Complex I
- Electron transport chain
- Complex II
- Mitochondrial membrane potential
- Malate-aspartate shuttle
- Energy utilising metabolic processes
- Glycolysis
- Gluconeogenesis
- (NAD/NADH NADP/NADPH) Co factors turnover
- Fructose
- Fructose metabolism
- Metabolic process
- in vitro assay
- Plasma metabolite

Putting Science to Work
### In vitro assay set

<table>
<thead>
<tr>
<th>Biochemical measurement</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I specific malate pyruvate oxidation</td>
<td>Flutamide, Rotenone</td>
</tr>
<tr>
<td>Complex II specific succinate oxidation</td>
<td>Carboxin</td>
</tr>
<tr>
<td>Mitochondrial membrane potential</td>
<td>Oligomycin, CCCP</td>
</tr>
<tr>
<td>Fatty acid synthase (FAS)</td>
<td>C75</td>
</tr>
<tr>
<td>Carnitine palmitoyl transferase 1 (CPT1)</td>
<td>MCoA, Amiodarone</td>
</tr>
<tr>
<td>Fatty acid influx (CD36)</td>
<td>Amiodarone</td>
</tr>
<tr>
<td>Microsomal triglyceride transferprotein (MTP)</td>
<td>Amiodarone</td>
</tr>
<tr>
<td>Gamma-γ-Glutamyl cysteine synthase (γGCS)</td>
<td>BSO</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>Carmastine</td>
</tr>
<tr>
<td>ROS generation</td>
<td>TBH</td>
</tr>
<tr>
<td>UGT assay</td>
<td>b-Napthoflavone (agonist), Diclofenac</td>
</tr>
<tr>
<td>Cellular ATP</td>
<td>Flutamide</td>
</tr>
<tr>
<td>Cellular GSH</td>
<td>BSO</td>
</tr>
<tr>
<td>Cellular TG</td>
<td>Gemfibrozil</td>
</tr>
<tr>
<td>Cell viability assay</td>
<td>Flutamide</td>
</tr>
</tbody>
</table>
**In vitro experimental protocol**

- HepG2 cells were used to measure cell viability at 24, 48 & 72 hrs
- 3 non-cytotoxic concentrations (>50% cell viability) were selected for each compound and detailed analysis was performed
- The response of the system to the drug has two components:
  - i. The response of individual enzyme (present in cell extract) to drug. Untreated cellular extract is used as source of enzyme and drug at required concentration is added directly into the reaction mixture to estimate the direct effect on enzyme activity ($f_d$).
  - ii. The adaptive response of the enzyme to the drug. Cells are treated with drugs, harvested and enzyme activity assessed ($f_a$).
- Pharmacokinetic parameters were used to estimate exposure
- Simulations were performed at normal human (1x) and 10x normal exposure-to assess the therapeutic window
- In the absence of drug-metabolism competence in HepG2 cell-system, the prediction is likely to be applicable only to the parent compound
BSO is a direct inhibitor of γ-GCS and a strong inducer of γ-GCS in treated cells.

The captured response of the enzyme to the drug has two components:

1) Untreated cellular extract is used as source of enzyme and drug at required concentration is added directly into the assay/reaction mixture to estimate the direct effect of drug on individual enzyme (fd).

2) The adaptive response of the system to the drug - Treated extract is used as source of enzyme to estimate adaptive effect on enzyme activity (fa).

53rd Annual Meeting of the Society of Toxicology, March 23–27, 2014
Simulation Protocol

• Altered enzyme activity due to drug treatment expressed as fold change against normal
• Drug exposure (concentration X time of treatment) vs. fold change in measured parameter is used as an input to the model
  • The total impact of the drug on the enzyme is given as \( f_d \times f_a \) where \( f_d \) is the fold-change due to direct effect of the drug on the enzyme and \( f_a \) is the adaptive fold-change in the enzyme level
• The drug effect on the system is simulated for several days and compared across various concentrations
• Key metabolites and fluxes are monitored to
  • assess the impact of drug on the system
  • understand the major processes responsible for drug impact either directly or in an adaptive manner
  • To elucidate the mechanism of action
All lab-measured parameters are input to the Virtual Liver together.
Some compounds successfully predicted by Heptox™

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>Tolcapone</td>
<td>Lapatanib</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Entacapone</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>AZT (azidodithymidine)</td>
<td>Atorvastatin</td>
<td>Ibuprofen</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>Nifedipine</td>
<td>Ranitidine HCl</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Ethinyl Estradiol</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Nimesulide</td>
<td>Troglitazone</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>Ximelagatran</td>
<td>Rosiglitazone</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Benazepril</td>
<td>Pioglitazone</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Methapyrilene</td>
<td>Tak875</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>Nicardipine (cardiotoxicant)</td>
<td>Metabolite of Tak875</td>
</tr>
<tr>
<td>Fenofibrate</td>
<td>Furosemide (cardiotoxicant)</td>
<td>Metformin</td>
</tr>
<tr>
<td>Perhexiline</td>
<td>Sorafenib (cardiotoxicant)</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>Metformine</td>
<td>CP-724714</td>
<td>Metabolite of Diclofenac</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Meloxicam</td>
<td>Zomepirac</td>
</tr>
<tr>
<td>Sulindac</td>
<td>Sudoxicam</td>
<td>BSO</td>
</tr>
</tbody>
</table>
## Prediction Accuracy of Heptox™

<table>
<thead>
<tr>
<th></th>
<th>Necrosis</th>
<th>Steatosis</th>
<th>Cholestasis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Accuracy (%)</strong></td>
<td>87</td>
<td>92</td>
<td>98</td>
</tr>
<tr>
<td><strong>True Positive Predicted (%)</strong></td>
<td>93</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td><strong>True Negatives Predicted (%)</strong></td>
<td>76</td>
<td>93</td>
<td>98</td>
</tr>
</tbody>
</table>
Case study

TAK875 is predicted to be associated with necrotic and cholestatic potential.
Complex I and II in electron transport chain (ETC) are inhibited equally by TAK875

- This may indicate a downstream target in ETC for causing membrane potential depolarization
- Complex I is also directly affected by the TAK875
TAK875 effects are simulated at different exposures

• Reported plasma drug concentration of TAK875 is 10 μM (1X)

• Simulations are performed at 1X, 2X, 5X and 10X of plasma drug concentration
80% depletion in cellular ATP observed at 5X exposure level will lead to necrotic death of cells
Reduction in rhodamine uptake with increase in exposure of TAK875 indicates depolarisation of mitochondrial membrane
Analysis of bile transporter data for TAK875 using Heptox™ model
Simulation strategy to interpret lab measured transporter activity

• All bile-acid and bilirubin transporters have been modeled with a Michaelis-Menten type kinetics

• We assume that all the experimental measurements have been performed with excess transporter substrate
  – Hence measured inhibition is assumed to be the same as transporter $V_{\text{max}}$ inhibition
**Schematic of Bile-Salt Transport**

**Induction of Cholestasis**

<table>
<thead>
<tr>
<th>Only Uptake Inhibition</th>
<th>Only Efflux Inhibition</th>
<th>Partial Inhibition of Both Uptake and Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum bile-salts will increase leading to greater excretion</td>
<td>Serum bile-salts will increase due to reflux leading to greater excretion</td>
<td>Serum bile-salts will increase leading to greater excretion</td>
</tr>
<tr>
<td>Intrahepatocellular bile-salts will reduce</td>
<td>Intrahepatocellular bile-salts will increase</td>
<td>Intrahepatocellular bile-salts may change either up or down depending upon the relative inhibition level</td>
</tr>
<tr>
<td>Canalicilar bile-salts will reduce</td>
<td>Canalicilar salt concentration will reduce</td>
<td>Canalicilar salt concentration will reduce</td>
</tr>
</tbody>
</table>
• The simulations have been performed considering 2 scenarios

  – TAK875 affects only the transporters alone (it is assumed that the drug has no other impact on the liver)

  – We consider the total impact of TAK875 on the transporters as well as on mitochondrial function
Scenario A: TAK875 Impact on transporters alone

At the highest dose of TAK875 - 50mM
- There is a transient increase in the flux of all the uptake transporters because of the large increase in serum bile salts to cholestatic levels\(^1\)
- BSEP flux shows 30% inhibition due to inhibition in Vmax; transient rise in intrahepatic bile salt is predicted before returning to normal

<table>
<thead>
<tr>
<th>Flux</th>
<th>Transient rise</th>
<th>Final Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTCP</td>
<td>~30%</td>
<td>Normal</td>
</tr>
<tr>
<td>OATP</td>
<td>~100%</td>
<td>~50% rise</td>
</tr>
<tr>
<td>BSEP</td>
<td>No change</td>
<td>~30% fall</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Transient Rise</th>
<th>Final Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA cytosol</td>
<td>~3 fold</td>
<td>Normal</td>
</tr>
<tr>
<td>TCA Serum</td>
<td>~11 fold</td>
<td>~8 fold</td>
</tr>
</tbody>
</table>

\(^1\) Arch Pharm Res Vol 29, No 4, 323-327, 2006
Scenario B: TAK875 affects transporters & Mitochondria

• TAK875 is predicted to lower ATP significantly (~ 80%) due to its mitochondrial impact

• Lowered ATP further reduces ATP mediated active transport via BSEP
  – 50% reduction in BSEP flux is predicted.

• This will lead to a larger increase in intracellular TCA almost 10 fold that remains for about 5 days before settling at 2-fold

• Similarly serum TCA will have a very high rise for about 5 days and remain at a sustained high of about ~10-fold
• TAK875 directly affects mitochondrial function leading to ATP depletion
• It also inhibits transporters leading to increase in intracellular bile salt
• As the ATP levels fall, the transporters activities are more severely affected leading to further TCA accumulation
• Accumulated bile salt over longer period of time may enhance mitochondrial dysfunction leading to further ATP depletion
  – Thus the two effects can potentiate each other leading to severe hepatocellular damage
TAK875 is predicted to be cholestatic

- ~8-10 fold change in serum TCA with large increase in serum bilirubin diglucuronide suggesting cholestasis
- Intracellular TCA level increases to very high levels when ATP mediated BSEP flux is inhibited due to the mitochondrial effects of the compound
- Since the mitochondrial injury seems to increase with time, increase in TCA levels probably cause further mitochondrial impairment.
Case study

Analysis of Troglitazone and Pioglitazone through Heptox™
Model simulation predicted Troglitazone associated necrotic and OS potential

Physiological average drug concentration
Troglitazone - 0.0006 mM (Dose 200 mg/day)
Pioglitazone - 0.0003 mM (Dose 15 mg/kg)

Simulated results are compared at 3 exposure levels - 1x (normal), 5x and 10x

Troglitazone and Pioglitazone-treated cultures: *In vitro* assays explain changes observed in end point measurements.

*In vitro measurement* using rat hepatocyte indicates inhibition in γ-GCS and mitochondrial complex I activity.
Can Heptox™ predict Idiosyncratic Toxicity?

A set of virtual patients are created with variation in BSEP activity (0-90% reduction in $V_{\text{max}}$ of BSEP transporter).

Simulations predict that individuals with >40% inhibition in BSEP expression are prone to cholestasis by Troglitazone treatment at 2.5X exposure (area highlighted in grey). *Plasma TCA level above 5 fold is considered as onset of cholestatic injury.*
Case study

SFN priming protects system from OS
Oxidative stress can lead to adverse responses including necrosis if a system is unable to adapt to its impact. Being able to quantitatively predict the response to oxidative stress will aid in the understanding of a chemical’s liability by improving our capability to define a tipping point between adaptive and adverse doses, or aid in the design of novel antioxidants.

We have developed a dynamic systems model of the biological processes involved in ROS generation & quenching, lipid peroxidation & protein oxidation, etc. Perturbing these pathways beyond the ability of NRF2- NFkB signaling to restore homeostasis culminates in oxidative stress induced cell death. We have combined the model with data generated in vitro on a set of enzymes predicted to be central in oxidative stress response including γ-GCS, GR, GPx, JNK, complexes I & II. All the enzyme activities were measured at different doses for a set of 20 compounds. A sub group was used for multi-parameter model optimization by monitoring various biomarkers such as MMP, ROS, nuclear NRF2 & NFkB, cellular MDA & 4HNE representing the progression of oxidative stress, and minimizing a cost function made of multiple experimentally measured values. The model was tested for its ability to predict the dose-dependent effect of curcumin that acts as antioxidant in some regimes and a pro-oxidant in others. We predict that low dose curcumin-primed cells can resist H$_2$O$_2$ and TBH induced oxidative stress compared to unprimed cells - a phenomena validated in vitro in HepG2 cells. We predict that at higher concentrations, curcumin induced cell-death originates from altered mitochondrial function. The ability to predict complex effects and system adaptations shows the value of integrating a validated systems model with the right biochemical assays - an approach that can predict the exposures likely to tip a system over from an adaptive regime causing adverse effects.
The Oxidative Stress Network

- Superoxide Formation cytosol
- Superoxide Formation mitochondria
- Cyto quenching by SOD, Catalase, GSH, TRx
- Mito quenching by SOD, GSH, PRX, TRx
- NO, peroxynitrite
- Quenching by protein thiols
- Lipid peroxidation Cyto membranes
- Lipid peroxidation Mito membranes
- Nrf2
- NFkB
- glycolysis
- Kreb's cycle
- Respiratory complexes
- Calcium
- JNK, ASK1, Caspase3
- Quenching by tocopherol, quinones
- Redox potentials
- Apoptosis
- Necrosis
- Mito pore
Platform Validation: Test compounds and *in vitro* assays

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Training set:</strong> Rotenone, Oligomycin, Flutamide, Troglitazone, Pioglitazone, Azathioprine, Hydrogen Peroxide, SIN-1, Doxorubicin, Menadione, DMNQ, tert butyl hydroperoxide, NAPQ1, Quercetin, tert-Butylhydroquinone, CDDO-Me, Tebufenpyrad</td>
<td>Cellular ATP, Mitochondrial membrane potential, Complex I activity, Complex II activity, Glutathione reductase activity, Gamma-glutamyl cysteine synthetase (gamma-GCS) activity, Glutathione peroxidase activity, Cellular GSH, Rate of cellular ROS generation, Cellular MDA, Cellular 4HNE, FoF1 activity, Mitochondrial ROS generation, Nuclear NRF2 transplocation, Cell viability (CCK assay)</td>
</tr>
<tr>
<td><strong>Test set</strong> Sulforaphane, Curcumin</td>
<td></td>
</tr>
</tbody>
</table>

**Training set:** Rotenone, Oligomycin, Flutamide, Troglitazone, Pioglitazone, Azathioprine, Hydrogen Peroxide, SIN-1, Doxorubicin, Menadione, DMNQ, tert butyl hydroperoxide, NAPQ1, Quercetin, tert-Butylhydroquinone, CDDO-Me, Tebufenpyrad

**Assays** Cellular ATP, Mitochondrial membrane potential, Complex I activity, Complex II activity, Glutathione reductase activity, Gamma-glutamyl cysteine synthetase (gamma-GCS) activity, Glutathione peroxidase activity, Cellular GSH, Rate of cellular ROS generation, Cellular MDA, Cellular 4HNE, FoF1 activity, Mitochondrial ROS generation, Nuclear NRF2 transplocation, Cell viability (CCK assay)
HepG2 cells are primed with various concentrations of SFN and Curcumin for 24h prior to introducing OS by

- Bolus treatment with 300 & 1000 mM H$_2$O$_2$ for 30 minutes (shorter treatment) or 120 minutes (longer treatment)
- Direct TBH treatment

ROS generation is measured as a direct effect of TBH

Cell response is analysed to understand the effect of priming in combating OS
• Transient reduction in GSH due to OS recovers faster in primed cells
• Model predicts higher level of nuclear NRF2 in SFN primed cells leading to increased GCS activity
HepG2 cells are primed with low and high concentration of SFN for o/n before TBH induced ROS generation is measured.
Case study

Analysis of the Cholestatic Mechanism of BI201335
BI 201335 is a drug that affects OATP, MRP2 and UGT1A1

BI 201335 effects are as follows\(^1\)

- MRP2: IC\(_{50}\) = 6.2 µM
- UGT1A1: IC\(_{50}\) = 0.5 µM
- OATP: IC\(_{50}\) = 0.6 µM

We translated this into corresponding suppression of the respective proteins

We reconstructed the PK curve using Table II\(^2\) and simulated 3 week therapy of BI 201335 in normal and Gilbert patients


Creation of Multiple Virtual Patients by Genetic knockdown

Table 1
Percentage composition of bilirubin, its mono- and diglucuronides present in bile of normal, Gilbert’s syndrome and Crigler-Najjar syndrome affected individuals

<table>
<thead>
<tr>
<th>Composition</th>
<th>Clinical state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Unconjugated bilirubin</td>
<td>1–3%</td>
</tr>
<tr>
<td>Bilirubin monoglucuronide</td>
<td>6–18%</td>
</tr>
<tr>
<td>Bilirubin diglucuronide</td>
<td>76–90%</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Bile Simulation</th>
<th>Normal</th>
<th>Gilbert Syndrome</th>
<th>CN2</th>
<th>CN1</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCB</td>
<td>2.2</td>
<td>7.3</td>
<td>26.2</td>
<td>100.0</td>
</tr>
<tr>
<td>BMG</td>
<td>14.8</td>
<td>19.1</td>
<td>25.9</td>
<td>0.0</td>
</tr>
<tr>
<td>BDG</td>
<td>83.0</td>
<td>73.5</td>
<td>48.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Putting Science to Work

BI201335 Simulation Reproduces Trial Results

**FIGURE 7.** Individual courses of total bilirubin in healthy volunteers treated for 21–28 days with 240 mg QD BI 201335, (A) without Gilbert’s disease, and (B) with Gilbert’s disease.
Features of Heptox™

• Assesses the risk of any compound in causing acute liver toxicity and identifies the probable mechanism involved by linking it to dose & exposure.
• Address 4 classical DILI end points: Necrosis, steatosis, cholestasis, oxidative stress
• Can identify safe and toxic regimes of exposure for any compound.
• Performs in vitro data to in vivo translation by simulation
• Can Integrate multiple inputs – in vitro data, microarray information, mitotox experiments, etc to provide a comprehensive look at a compound’s impact
Thank you