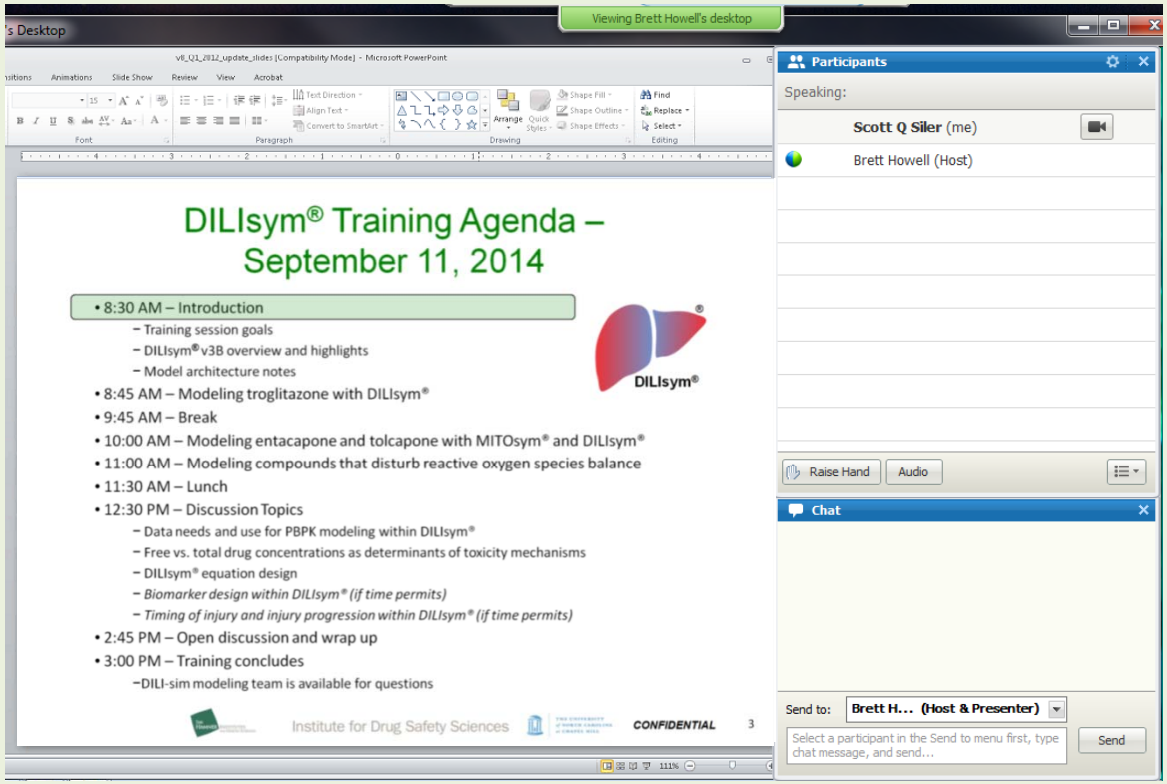
WebEx Tools will be Used to Record this Training Session

- Recording meeting for future reference and members not able to attend



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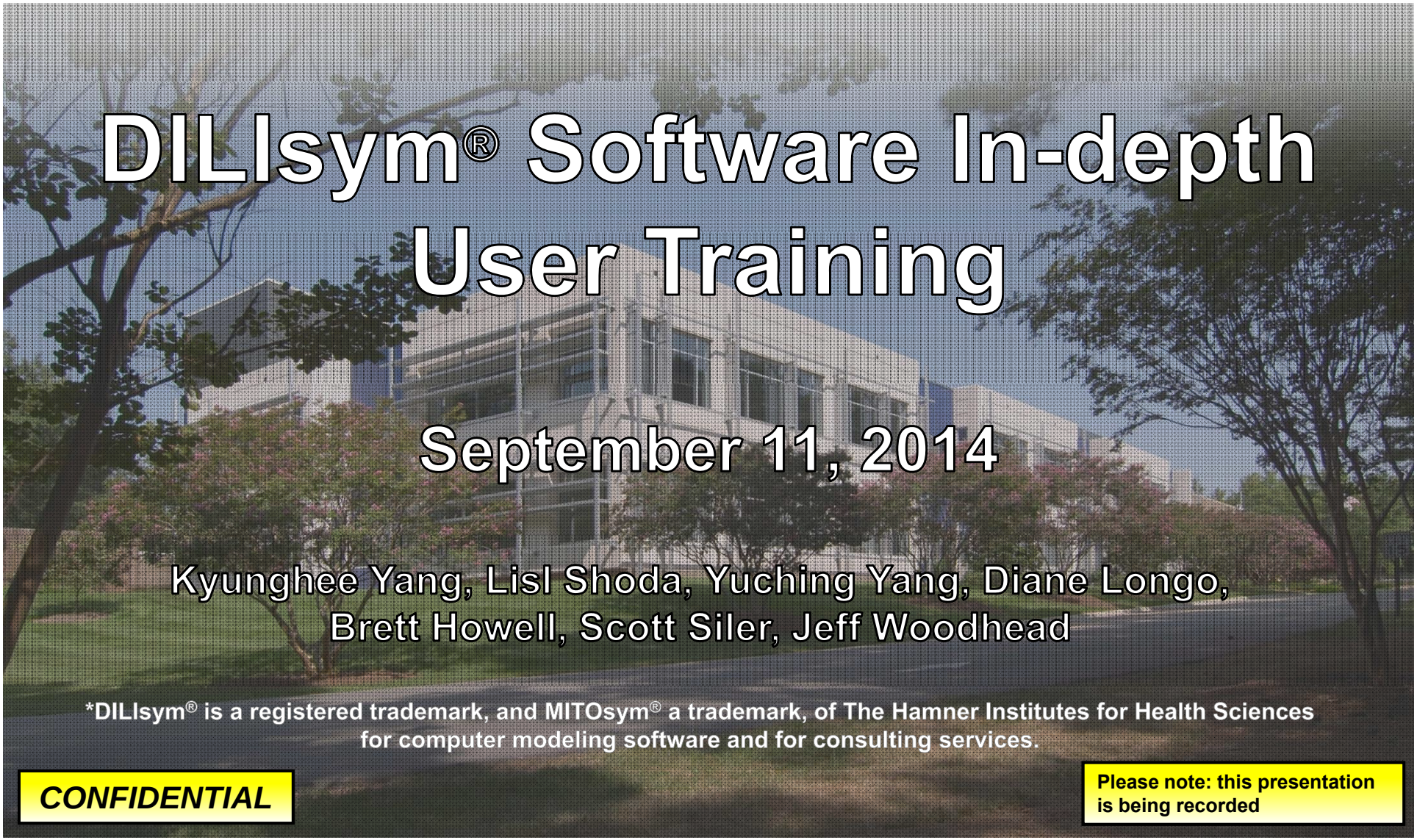


DILIsym® Software In-depth User Training

September 11, 2014

Kyunghee Yang, Lisl Shoda, Yuching Yang, Diane Longo,
Brett Howell, Scott Siler, Jeff Woodhead

DILIsym® Training Agenda – September 11, 2014

- 8:30 AM – Introduction
  - Training session goals
  - DILIsym® v3B overview and highlights
  - Model architecture notes
- 8:45 AM – Modeling troglitazone with DILIsym®
- 9:45 AM – Break
- 10:00 AM – Modeling entacapone and tolcapone with MITOsym® and DILIsym®
- 11:00 AM – Modeling compounds that disturb reactive oxygen species balance
- 11:30 AM – Lunch
- 12:30 PM – Discussion Topics
  - Data needs and use for PBPK modeling within DILIsym®
  - Free vs. total drug concentrations as determinants of toxicity mechanisms
  - DILIsym® equation design
  - *Biomarker design within DILIsym® (if time permits)*
  - *Timing of injury and injury progression within DILIsym® (if time permits)*
- 2:45 PM – Open discussion and wrap up
- 3:00 PM – Training concludes
  - DILI-sim modeling team is available for questions

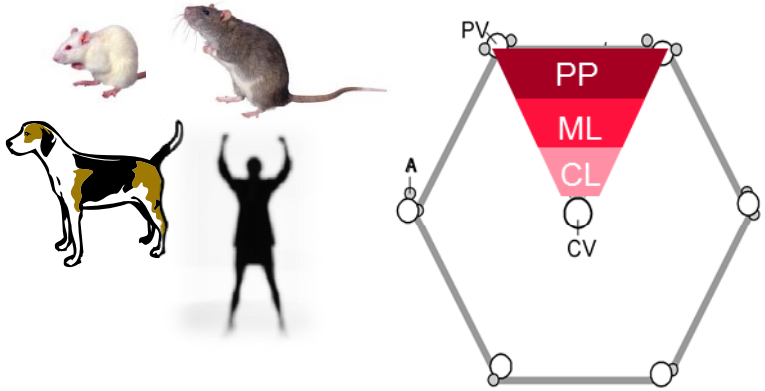
Goals for the Q3 2014 DILIsym® In-depth User Training Session

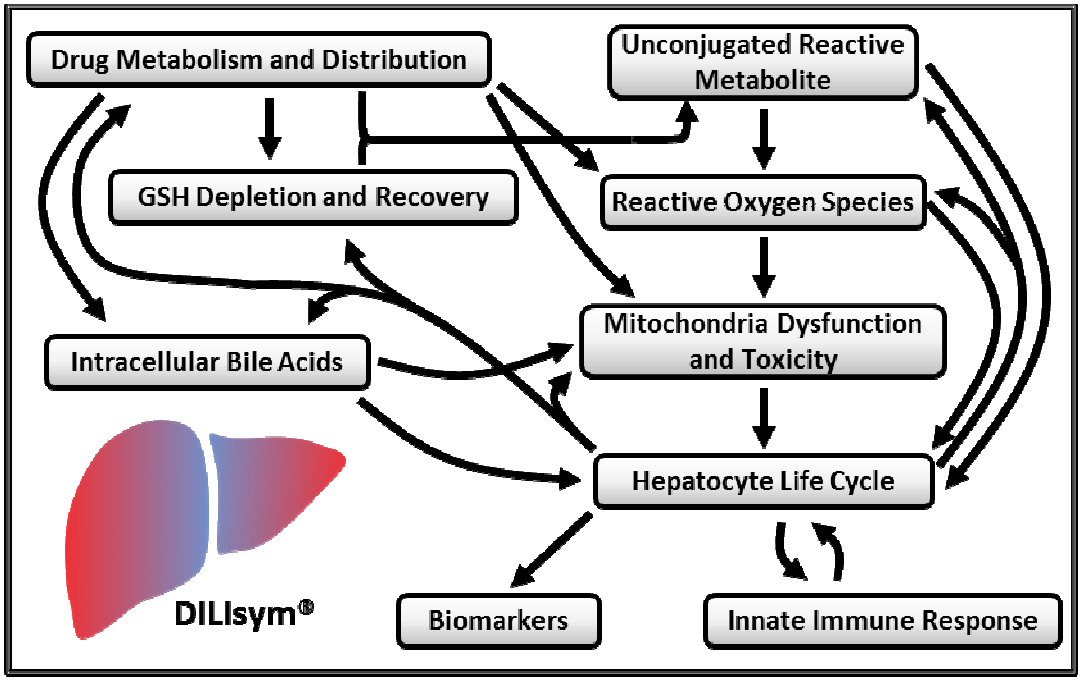
Participants should understand the following general concepts:

- The conceptual model architecture of DILIsym®
- Key updates and changes for DILIsym® v3B, the most recent release
- The concept of “translatability” as it applies to DILIsym®
- Data types and methods necessary to simulate bile acid transport disruption in DILIsym®
- Data types and methods necessary to simulate disruption of mitochondrial function in DILIsym®
- Data types and methods necessary to simulate disturbances in the reactive oxygen species balance in DILIsym®
- The approaches generally taken by the DILIsym® development team to construct PBPK models using various data types
- The approach taken in the current versions of DILIsym® regarding free versus total drug concentrations for inducing DILI
- The general approach taken by the DILIsym® development team to construct equations within DILIsym®

DILIsym® Overview

- **Multiple species: human, rat, mouse, and dog**
  - Population variability
- **The three primary acinar zones of liver represented**
- **Essential cellular processes represented to multiple scales in interacting sub-models**
  - Pharmacokinetics
  - Dosing (IP, IV, Oral)
  - Transporter Inhibition
  - Drug metabolism
  - GSH depletion
  - Injury progression
  - Mitochondrial dysfunction, toxicity
  - Bile acid mediated toxicity
  - Cellular energy balance
  - Hepatocyte life cycle
  - Macrophage, LSEC life cycles
  - Immune mediators
  - Caloric intake
  - Biomarkers





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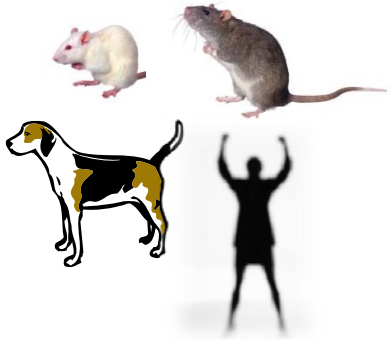
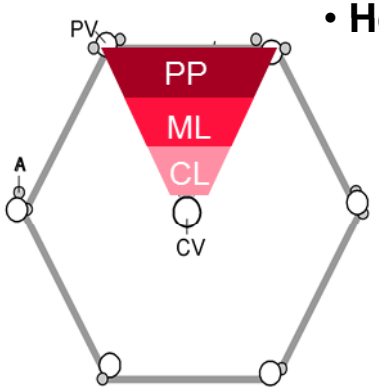
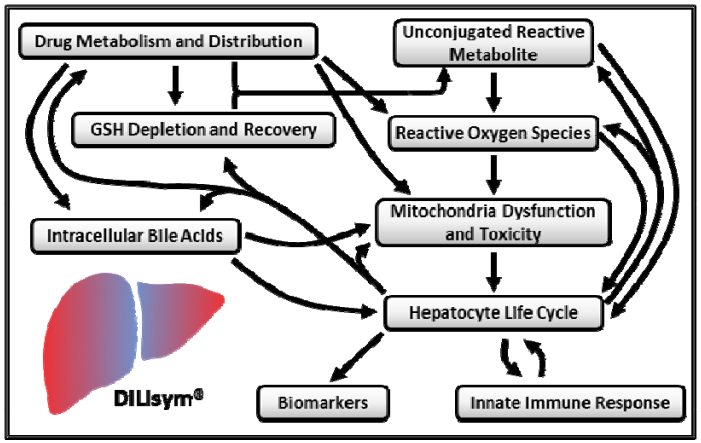




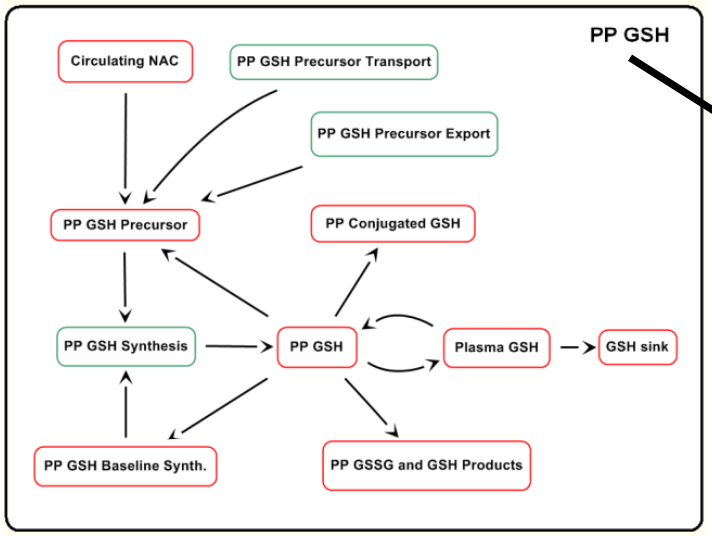


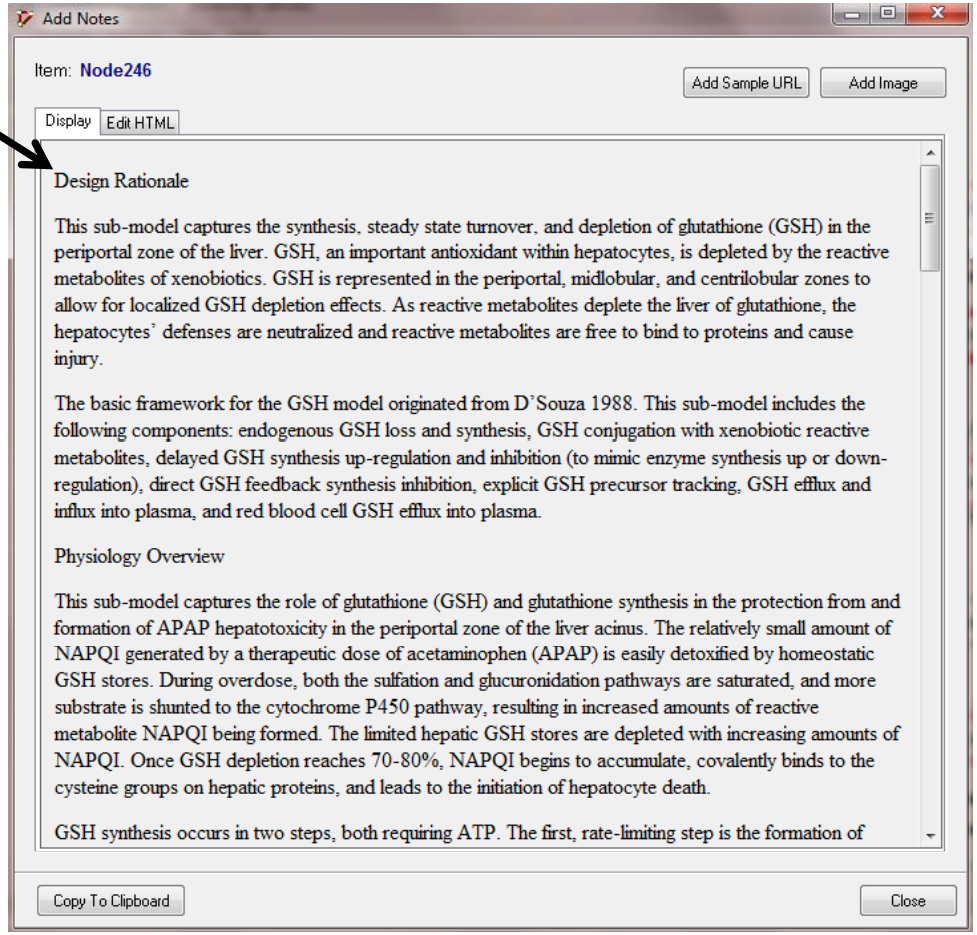
- **Compartment-based modeling**
  - >500 state variables
  - 'Form to function' connection
  - Ordinary differential equations
  - Code or GUI functionality

- **Hepatotoxicity exemplars**
  - Reactive metabolite mediated
    - Acetaminophen
    - Methapyrilene
    - Furosemide
    - Aflatoxin B1
    - Carbon tetrachloride
  - Mitochondrial dysfunction
    - Etomoxir
    - Buprenorphine
    - Tolcapone
    - CP-724714
  - Bile acid transporter inhibition
    - Glibenclamide
    - CP-724714
    - Bosentan
    - Telmisartan
    - Tolcapone
    - Troglitazone
    - Pioglitazone
  - Single, multiple dose protocols
  - Single, combination drug protocols

DILIsym® Qualitative Diagram and Documentation

- Illustrates mechanisms with corresponding documentation



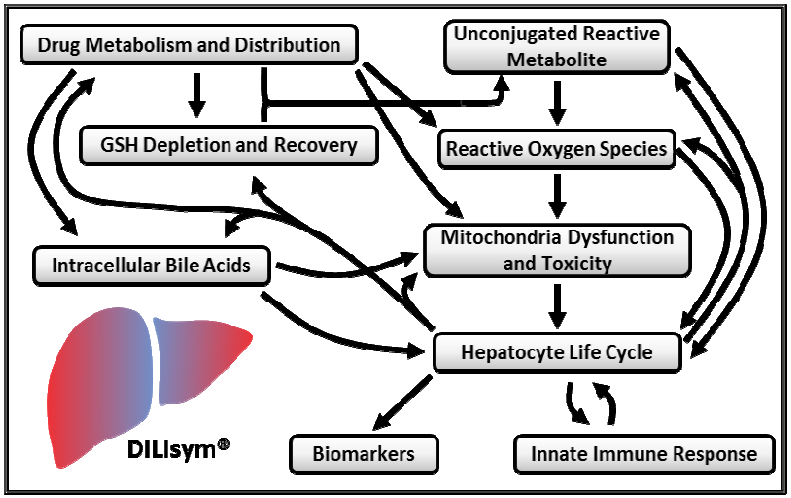


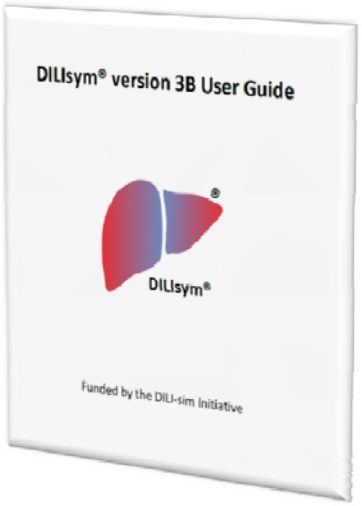
J-Designer
Systems Biology Workbench

http://sys-bio.org/

Sauro Lab, University of Washington

Highlights of DILIsym® v3B



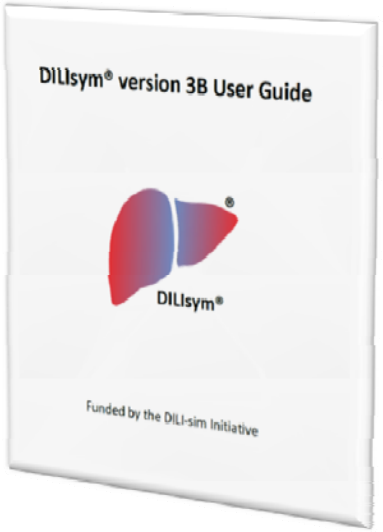


- Added apoptosis as an active mode of cell death during hepatotoxicity
- Added caspase-cleaved cytokeratin 18 as a biomarker indicative of apoptosis
- Expanded representation of existing biomarkers to include apoptosis
- Added inhibition of glycolysis as a mechanism of hepatotoxicity
- Various bug fixes

- Introduced additional exemplar compounds for exposure-related toxicity
  - Troglitazone
  - Pioglitazone
- Additional SimPops™, capturing impact of variability in key pathways
  - SimPops™ combining oxidative stress, mitochondrial dysfunction, and caspase activation variability
  - SimPops™ focused on troglitazone (humans and rats) and pioglitazone (humans)

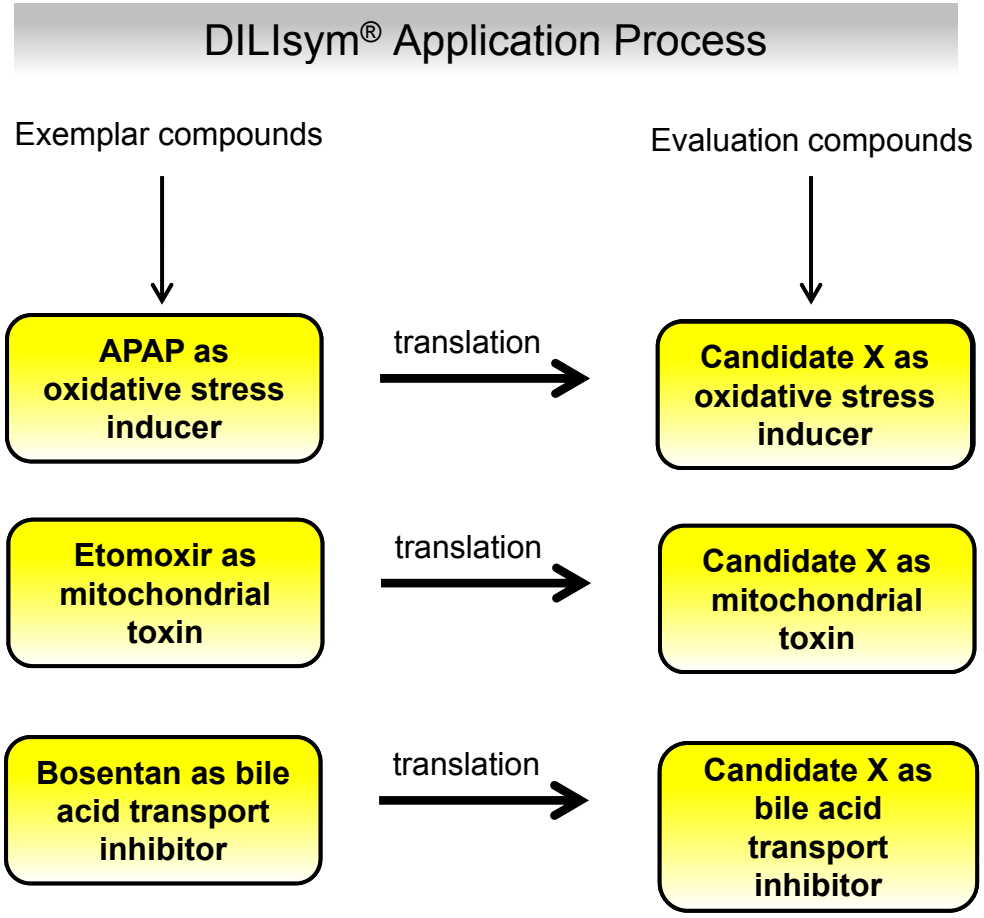
Expanded Capabilities and Features of DILIsym® v3B

- *Added* apoptosis as an active mode of cell death during hepatotoxicity
  - Apoptosis is induced via ROS/RNS increases
- *Expanded* representation of existing biomarkers to include apoptosis
  - Biomarkers are released during apoptosis differently depending on the number of dead hepatocytes in the liver
- *Added* more pre-equilibrated sets of state variables for SimPops™ to save simulation time
- *Expanded* the documentation on data used for SimPops™ development
- *Updated* the parameter overrides feature to include an error check that prevents incorrect parameter spellings from going undetected
- *Expanded* Zotero reference database (contact us for real-time access)
- Altered commands used to utilize parallel processing in MATLAB
  - Dictated by a MATLAB change in MATLAB 2013b
  - **DILIsym® v3B will only function properly with MATLAB 2013b and newer versions (not MATLAB 2013a or older)**
- Various bug fixes
  - Active transport representation for liver uptake of drugs was updated to include normalization based on viable hepatocytes
  - Various passive transport pathways for drugs and endogenous molecules were updated to include normalization based on changes in viable hepatocytes
  - The ROS/RNS generation mechanism for Compound W was updated to remove a coding error



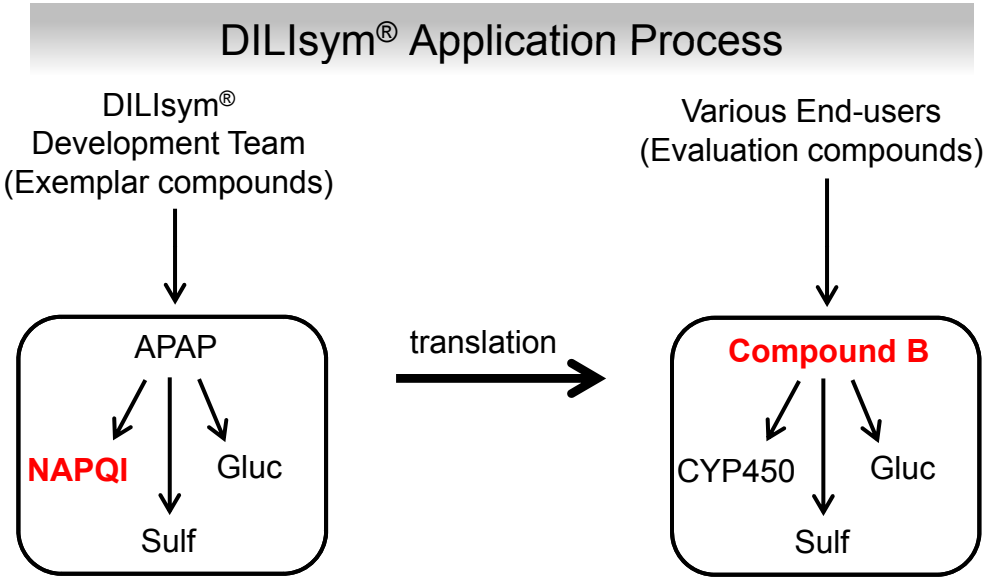
DILIsym® Architecture – Translation from Exemplar Compounds to Compounds of Interest

- The value proposition of DILIsym® lies in its ability to translate to compounds NOT used to build it
- This requires end-users with evaluation compounds to either have an idea of what mechanisms of hepatotoxicity might be in play or conduct hypothesis-based modeling
- Multiple, concurrent mechanisms of hepatotoxicity can be used and are being explored



DILIsym® Architecture – Using the Mechanism Selection Tool

- The mechanism selection tool allows the end-user to select an existing mechanism in the DILIsym® model
- Importantly, the tool also allows the mechanism to be applied anywhere in the metabolism tree
- The user can also apply multiple mechanisms to the same chemical species and different mechanisms to different levels of the tree
  - Parent and metabolite with same mechanism
  - Parent and metabolite with different mechanisms



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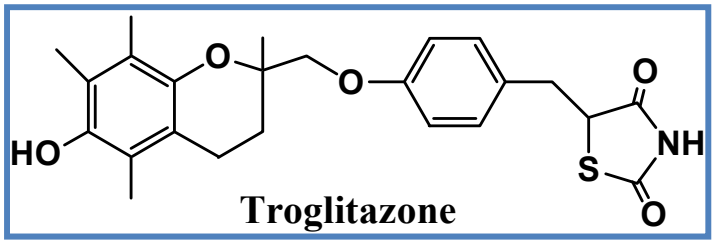
Modeling Compounds that Inhibit Bile Acid Transport: A Case Study with Troglitazone

- Introduction
  - Troglitazone effects on hepatic bile acid transporters
  - Structure of bile acid transport inhibition section within DILIsym®
- Modeling troglitazone-mediated hepatotoxicity that involves bile acid transport inhibition
  - Gathering data inputs for bile acid transport inhibition
  - Translate data to DILIsym® parameters
  - Construction of a PBPK model
- Simulate troglitazone-mediated hepatotoxicity using DILIsym®
  - Simulate troglitazone-mediated hepatotoxicity in baseline human and rat
  - Construction of human and rat bile acid Simpops™
  - Simulate troglitazone-mediated hepatotoxicity using human and rat SimPops™

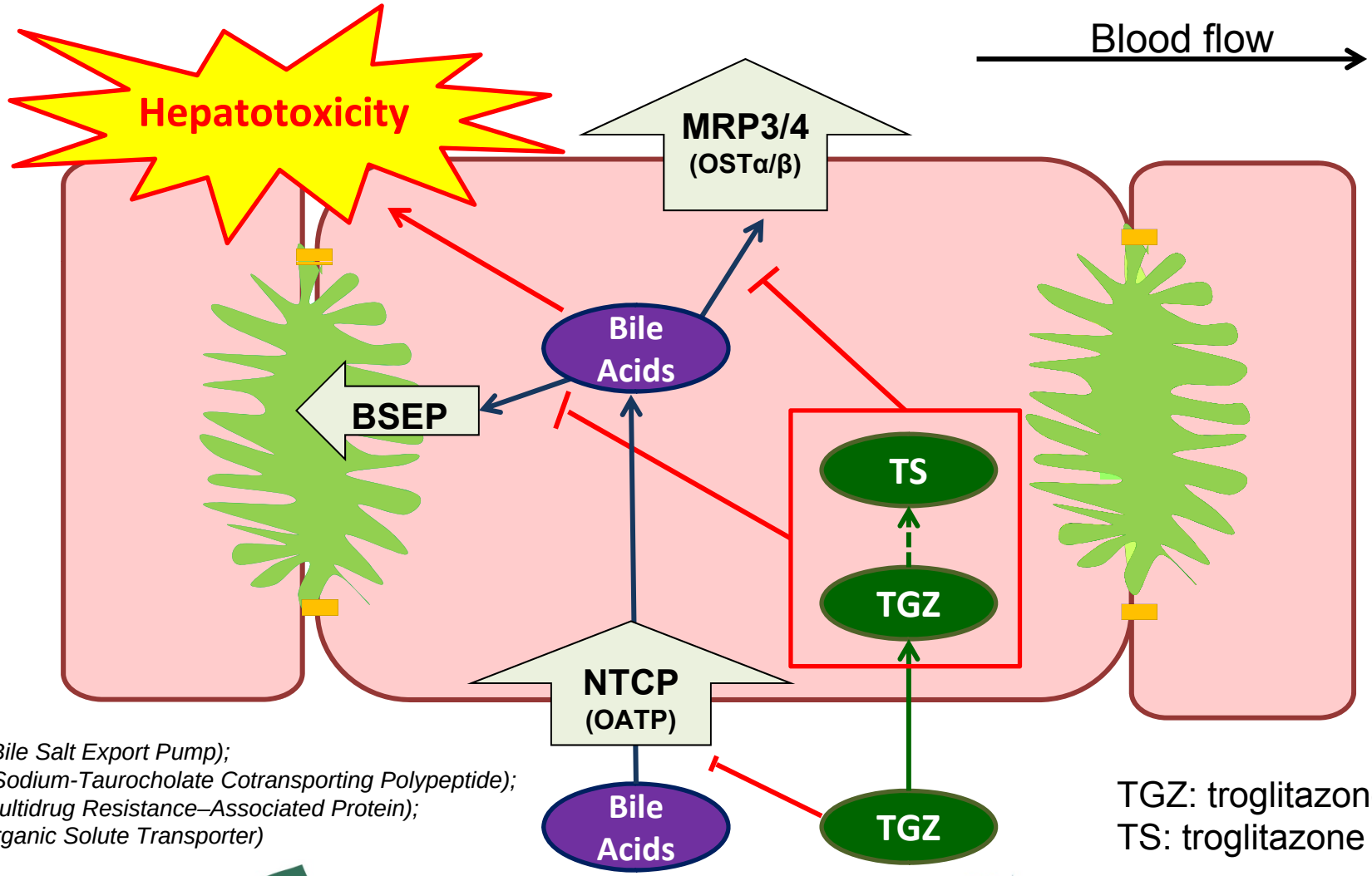
Troglitazone (TGZ)



- First in thiazolidinedione class; PPARγ agonist
  - Reduces hepatic and peripheral insulin resistance
  - Approved for the treatment of type II diabetes

- Hepatotoxicity
  - Hepatotoxicity was not detected in preclinical studies
  - 2% of patients developed ALT elevations >3X ULN in clinical trials
  - Withdrawn from the market due to idiosyncratic hepatotoxicity

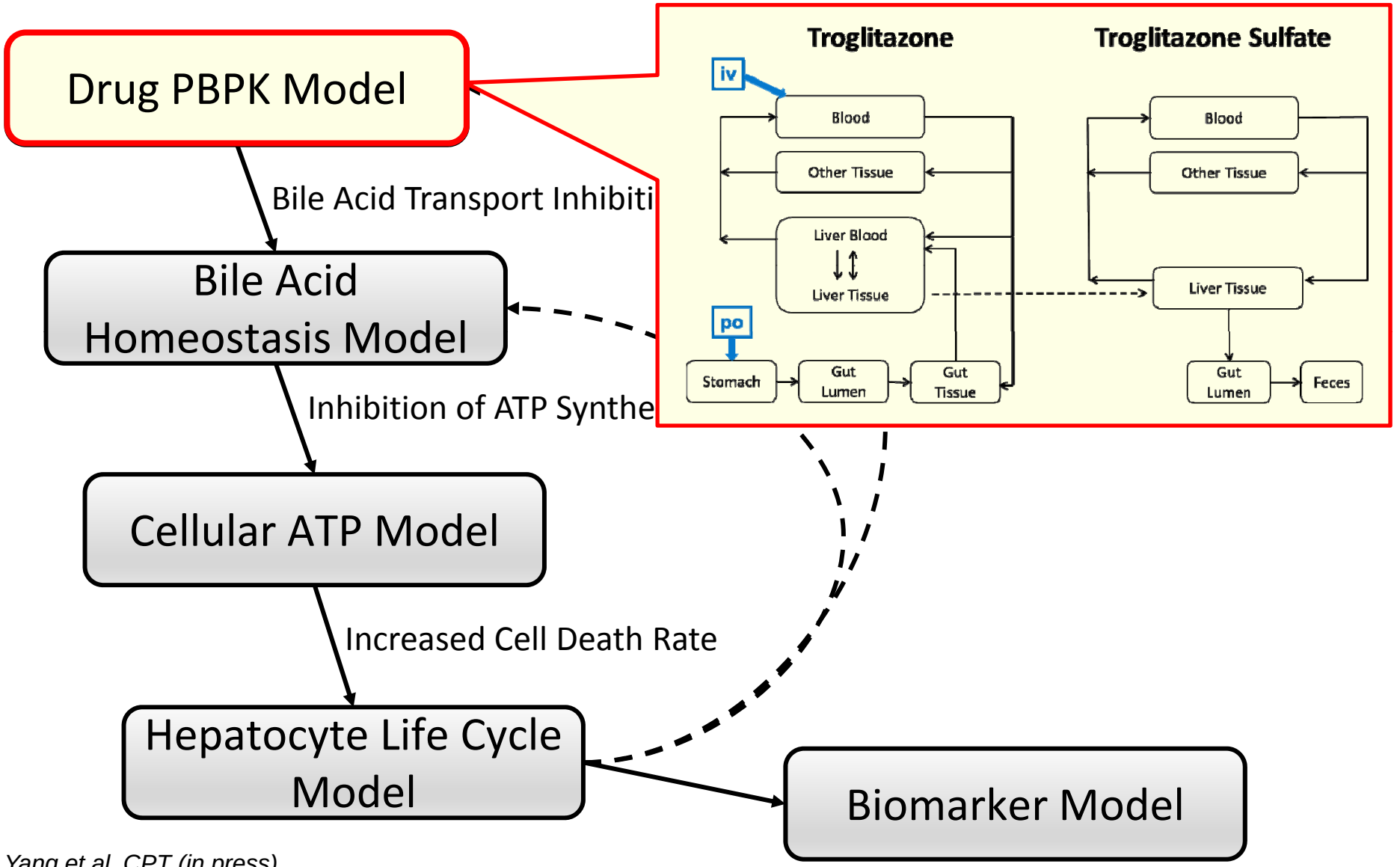
Mechanisms of DILI: Transport Protein-Mediated Bile Acid-Drug Interaction



BSEP (Bile Salt Export Pump);
NTCP (Sodium-Taurocholate Cotransporting Polypeptide);
MRP (Multidrug Resistance–Associated Protein);
OST (Organic Solute Transporter)

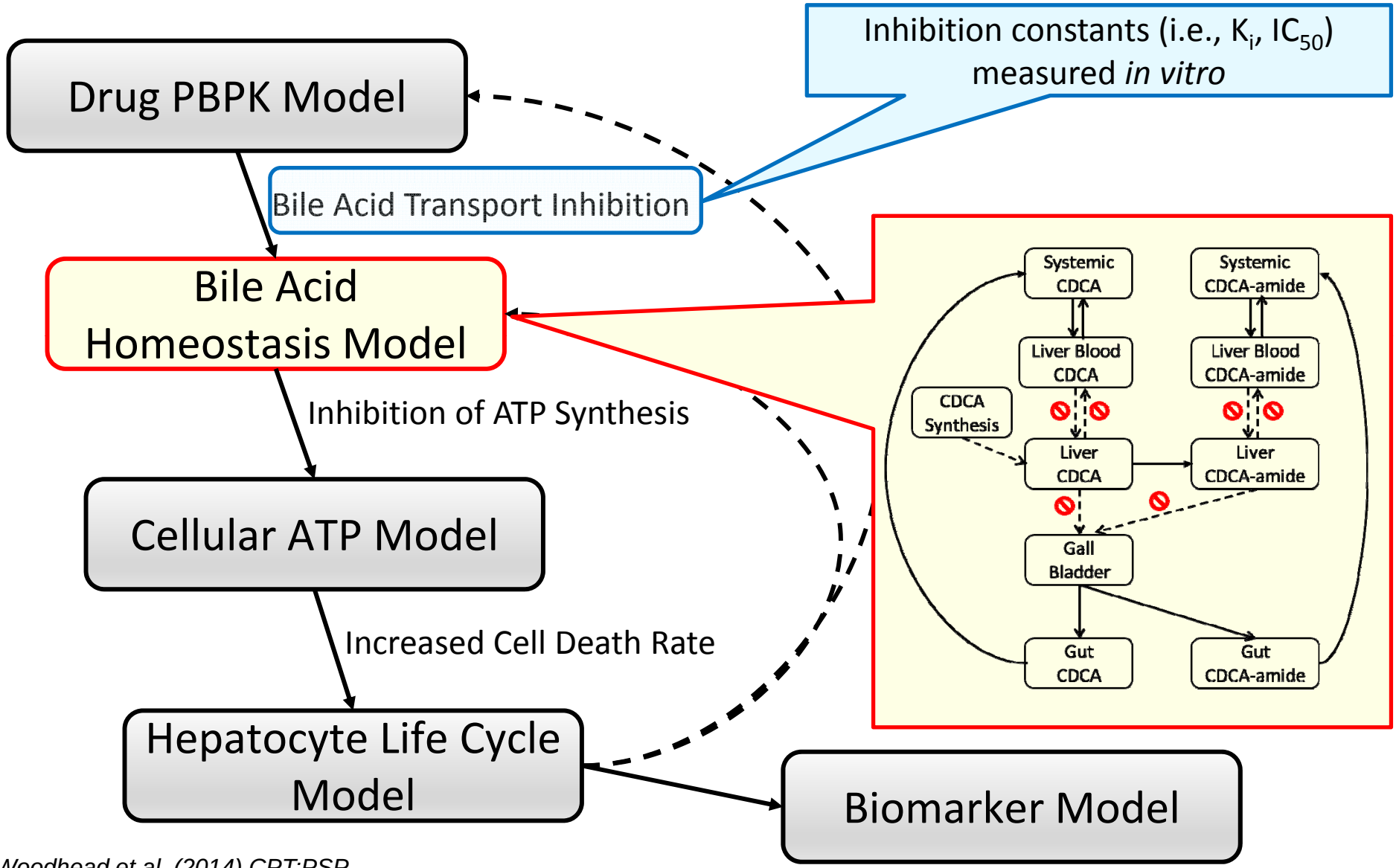
TGZ: troglitazone
TS: troglitazone sulfate

Bile Acid Transport Inhibition Section



Yang et al. CPT (in press)

Bile Acid Transport Inhibition Section



Woodhead et al. (2014) CPT:PSP
Yang et al. CPT (in press)

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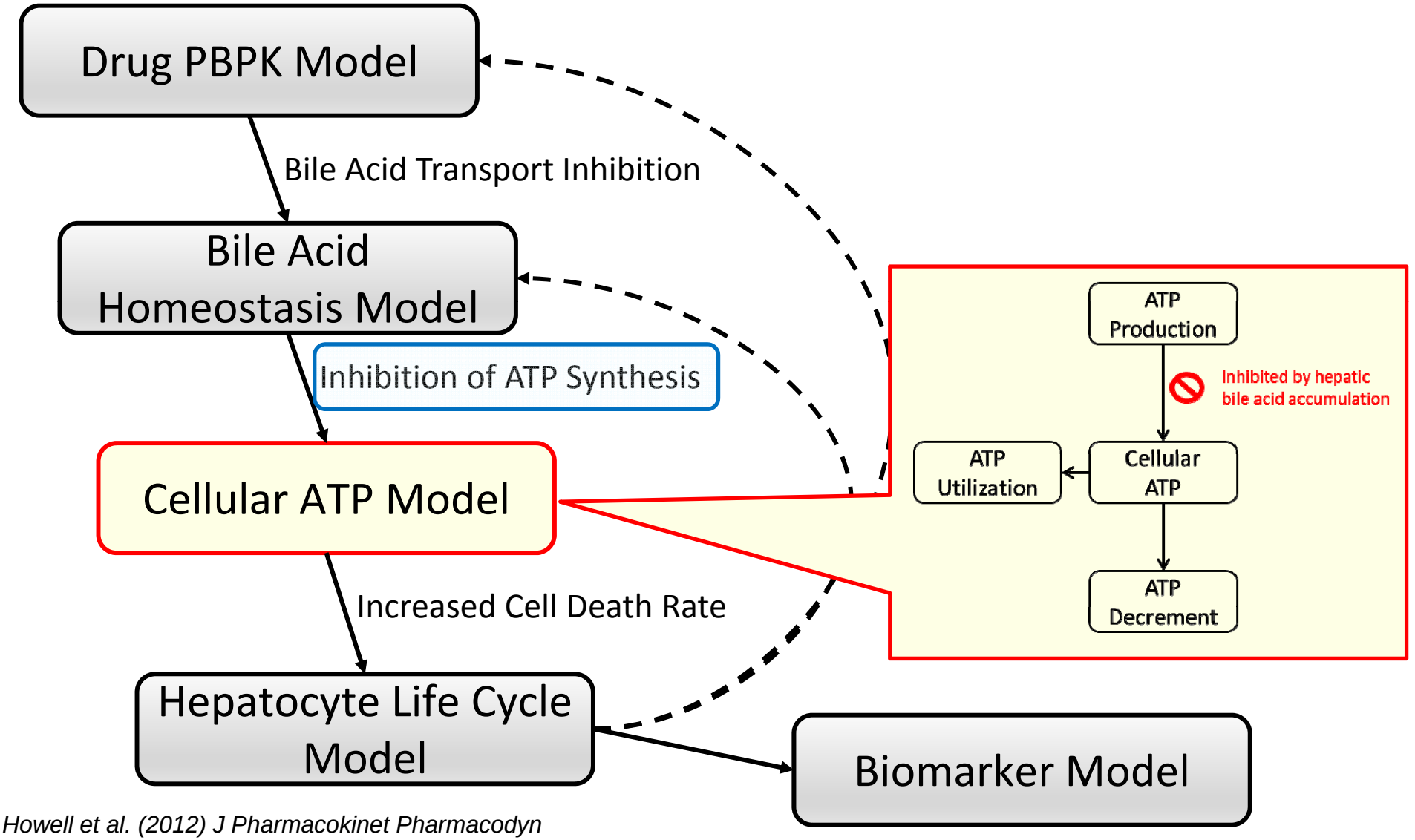

Howell et al. (2012) J Pharmacokinet Pharmacodyn
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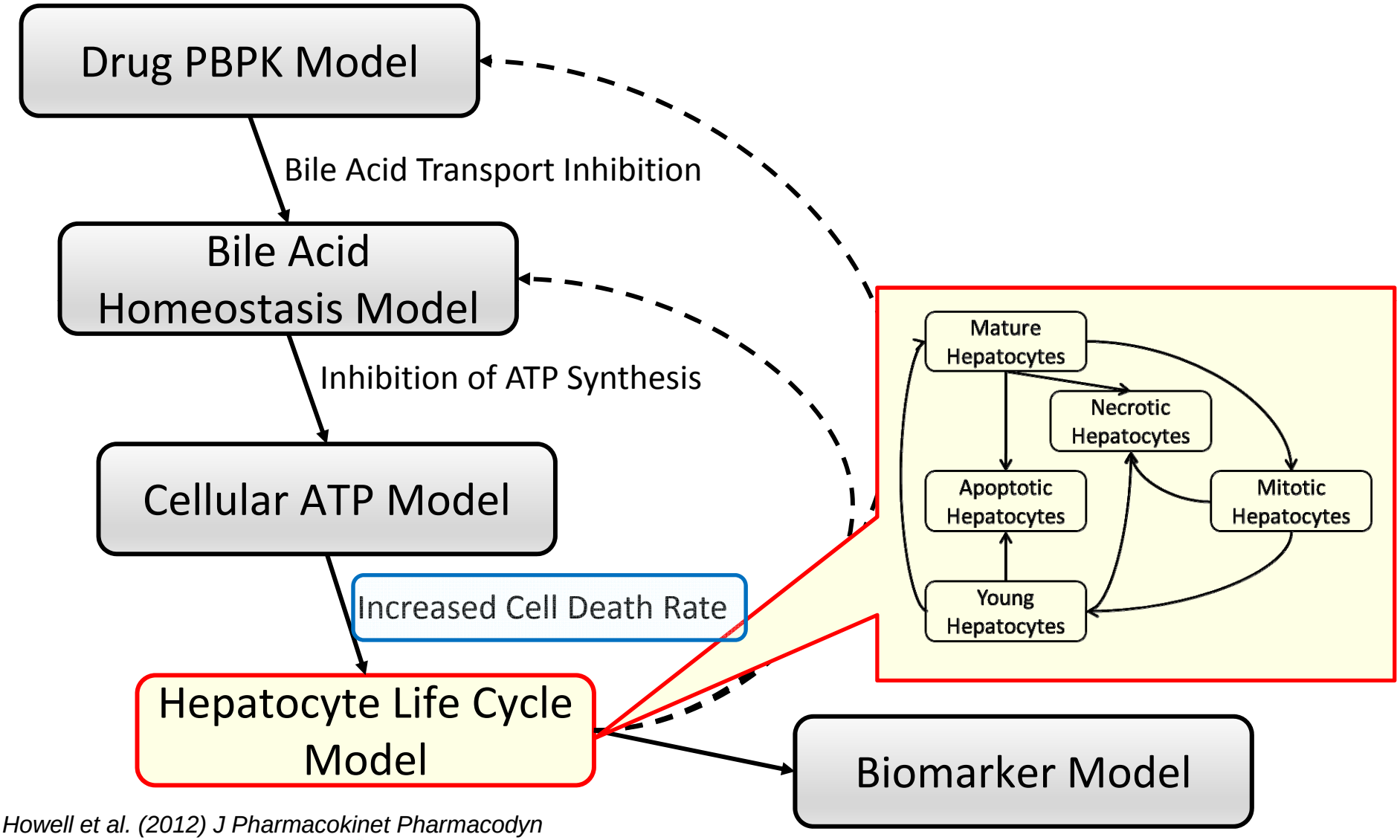

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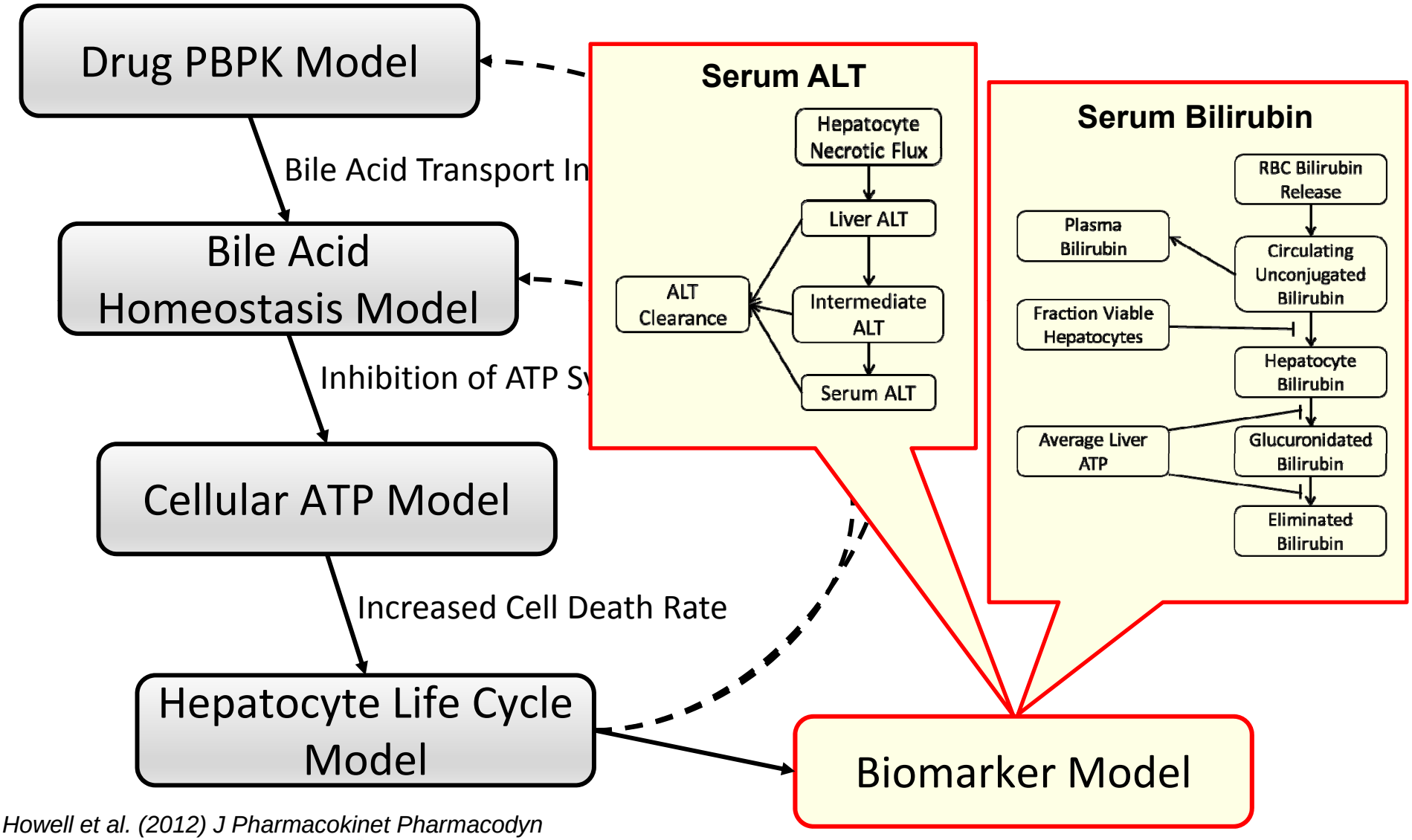

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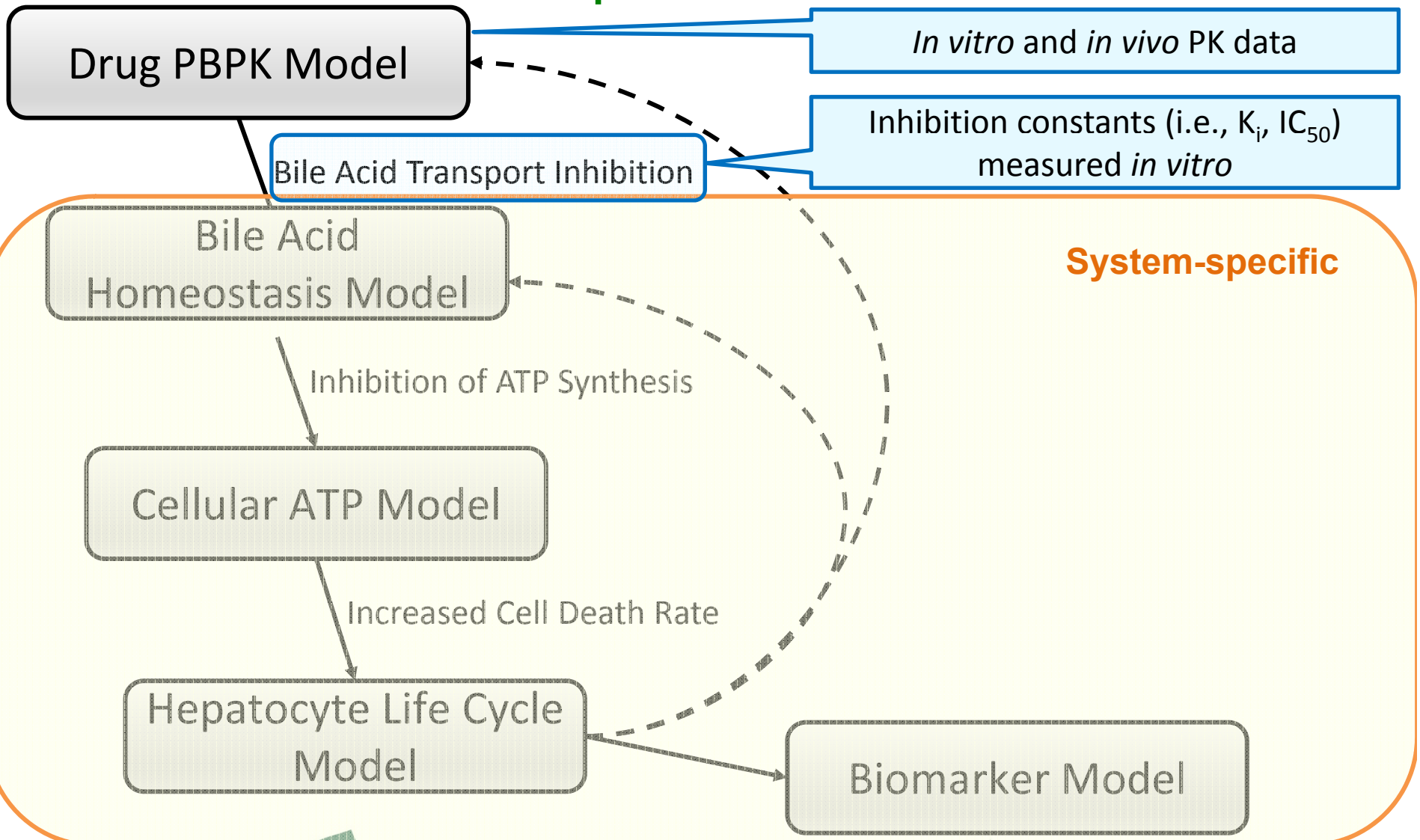
Gathering Data Inputs for Bile Acid Transport Inhibition



Gathering Data Inputs for Bile Acid Transport Inhibition

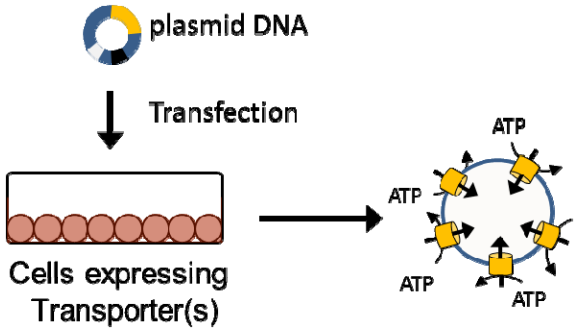
- Data inputs
  - Inhibition constant: K_i, IC_{50}
  - Type of inhibition: competitive, noncompetitive, uncompetitive, mixed
- *In vitro* assessment using multiple bile acid transporters recommended

Transporter	Function	Experimental System
BSEP	Biliary excretion	Membrane vesicles
MRP3, MRP4	Basolateral efflux	Membrane vesicles
NTCP	Basolateral uptake	Primary hepatocytes, transfected cell lines

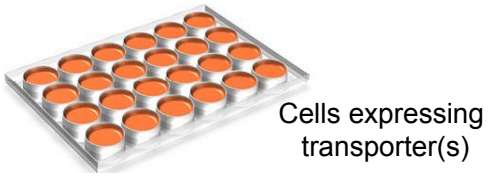
- Assessment of inhibitory effects of stable metabolites
  - If systemic/hepatic exposure of stable metabolites are high, incorporation of metabolite effects provide more reliable predictions (e.g., troglitazone sulfate)
  - Identification and synthesis of stable metabolites are not feasible during early stages of drug development

In vitro Systems to Assess Bile Acid Transport Inhibition

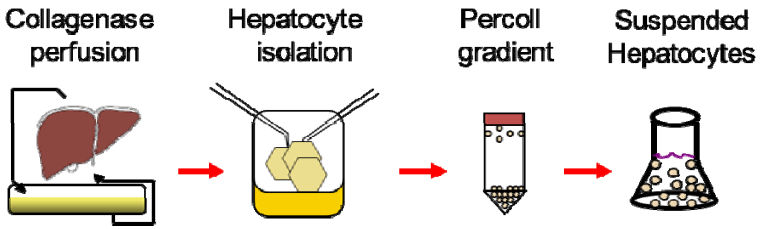
- Membrane vesicles
  - Used to assess efflux transporters (e.g., BSEP, MRP3, MRP4)
  - Source: liver plasma membrane, transfected cells (e.g., Sf9, Sf21)



- Transfected cell lines
  - Used to assess uptake transporters (e.g., NTCP)



- Primary hepatocytes
  - Suspended hepatocytes can be used to assess hepatic uptake
  - Can assess sodium-dependent and sodium-independent transport
  - Costly



In vitro Methods to Assess Bile Acid Transport Inhibition

Inhibition constant	IC_{50}	K_i
Definition	Inhibitor concentration at the half maximal activity	Affinity of the inhibitor to the probe substrate binding site
Experimental methods	Transport assays with one substrate concentration & multiple inhibitor concentrations	Transport assays with multiple substrate concentrations & multiple inhibitor concentrations
Robustness	Varies depending on the substrate concentrations IC_{50} will approach Ki, if [S] << K_m	A more robust parameter
Provide information on the type of inhibition?	No	Yes
Cost	$	$$$
Comment	Commonly measured	Recommended for reliable prediction of hepatotoxicity

Translate Bile Acid Transport Inhibition Data to DILIsym® Parameters: Troglitazone

- Troglitazone competitively inhibits rat Bsep with an Ki of 1.3 μM†
  - Will use this value for humans too; literature has shown that troglitazone has similar potency for rat and human BSEP
- Troglitazone inhibits human NTCP and rat Ntcp‡
  - IC_{50} values reported: 0.33 μM (human), 2.3 μM (rat)
  - Type of inhibition not known; assumed to be a competitive inhibitor
- Troglitazone is an inhibitor of human MRP4§
  - IC_{50} measured: 21 μM
  - Type of inhibition not known; assumed to be a non-competitive inhibitor

Troglitazone m.w. 441.5 g/mol

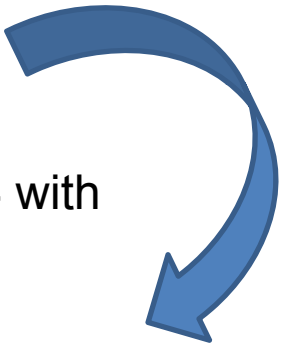
DILIsym® Parameter Name	DILIsym® Parameter Description	DILIsym® Parameter Input
Ki_BSEP_CompX	Competitive Ki for BSEP; parent Compound X	5.74E-04 mg/mL
Ki_NTCP_CompX	Competitive Ki for NTCP; parent Compound X	1.46E-04 mg/mL (human); 1.02E-03 mg/mL (rat)
Ki_noncom_baso_CompX	Noncompetitive Ki for basolateral efflux; parent Compound X	9.27E-03 mg/mL

†Funk 2001, Dawson 2011, ‡Marion 2007, §Yang in preparation

Translate Bile Acid Transport Inhibition Data to DILIsym® Parameters: Troglitazone Sulfate

- Troglitazone sulfate is a more potent inhibitor of BSEP compared to troglitazone†
  - Troglitazone sulfate competitively inhibits rat Bsep with an Ki of 0.23 µM
  - Will use this value for humans too
- Troglitazone sulfate effects on NTCP not known
  - Assumed to be the same as troglitazone‡
- Troglitazone sulfate is a non-competitive inhibitor of human MRP4 with an Ki of 8 µM§
  - Rat Mrp4 Ki is assumed to be the same as humans

Troglitazone sulfate
m.w. : 521.6 g/mol



DILIsym® Parameter Name	DILIsym® Parameter Description	DILIsym® Parameter Input
Ki_BSEP_CompX_MetB	Competitive Ki for BSEP; Compound X Metabolite B	1.20E-04 mg/mL
Ki_NTCP_CompX_MetB	Competitive Ki for NTCP; Compound X Metabolite B	1.46E-04 mg/mL (human); 1.02E-03 mg/mL (rat)
Ki_noncom_baso_CompX_MetB	Noncompetitive Ki for basolateral efflux; Compound X Metabolite B	4.17E-03 mg/mL

†Funk 2001, Dawson 2011, ‡Marion 2007, §Yang in preparation

The PBPK Representation in DILIsym® Depends Heavily on the Development Stage of the Compound being Simulated

Early candidate screening

- Metabolic clearance of parent compound and coincidental appearance of specific metabolites *in vitro*
- Potential for active transport in the liver *in vitro* (rate of hepatocyte uptake); transport kinetic information if possible
- Basic molecular properties
  - Acid or base?
  - Monoprotoic or diprotic
  - pKa(s)
  - log P (oil:water and octanol:water)
  - Fraction bound to plasma or serum proteins
  - Fraction partitioned into red blood cells

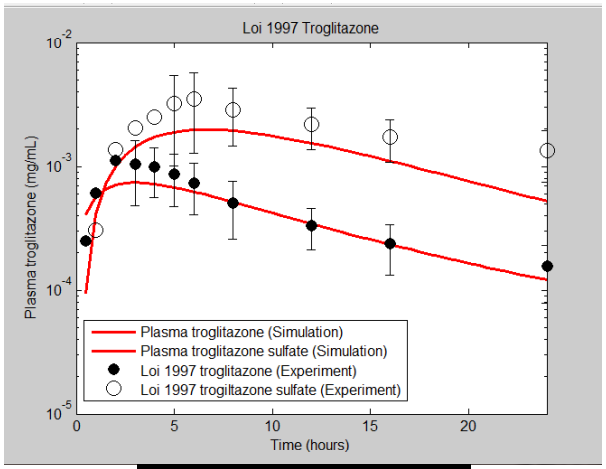
Late-stage development / OTM

- *In vivo* PK time-course and dose-response
- Mass balance tissue distribution studies in animals (*in vitro* accumulation as well)
- *In vitro* drug metabolism assays identifying the appropriate metabolizing enzymes for the drug
- Metabolic clearance of parent compound and coincidental appearance of specific metabolites
- Potential for active transport in the liver (rate of hepatocyte uptake); transport kinetic information if possible
- Basic molecular properties

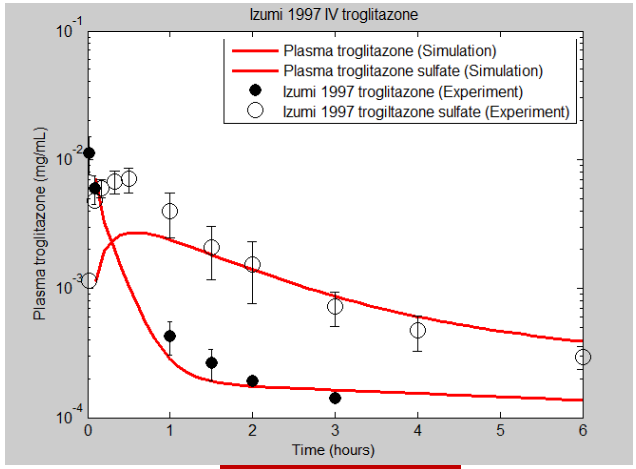
TROGLITAZONE

PBPK Modeling: Troglitazone and Troglitazone Sulfate Disposition

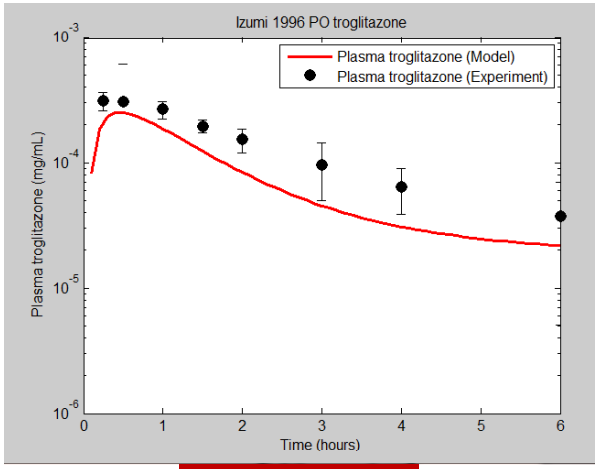
- Troglitazone is extensively metabolized to troglitazone sulfate (TS) in humans and rats
  - Vmax and Km values for the formation of TS and troglitazone glucuronide (TG) reported
  - TG and TS were represented by Metabolite A and Metabolite B, respectively
- TS PBPK model was constructed
  - TS is a potent inhibitor of bile acid transporters
  - TG PBPK model was not constructed because TG is a minor metabolite and its effects on bile acid transporters are not known



HUMANS (PO)



RATS (IV)



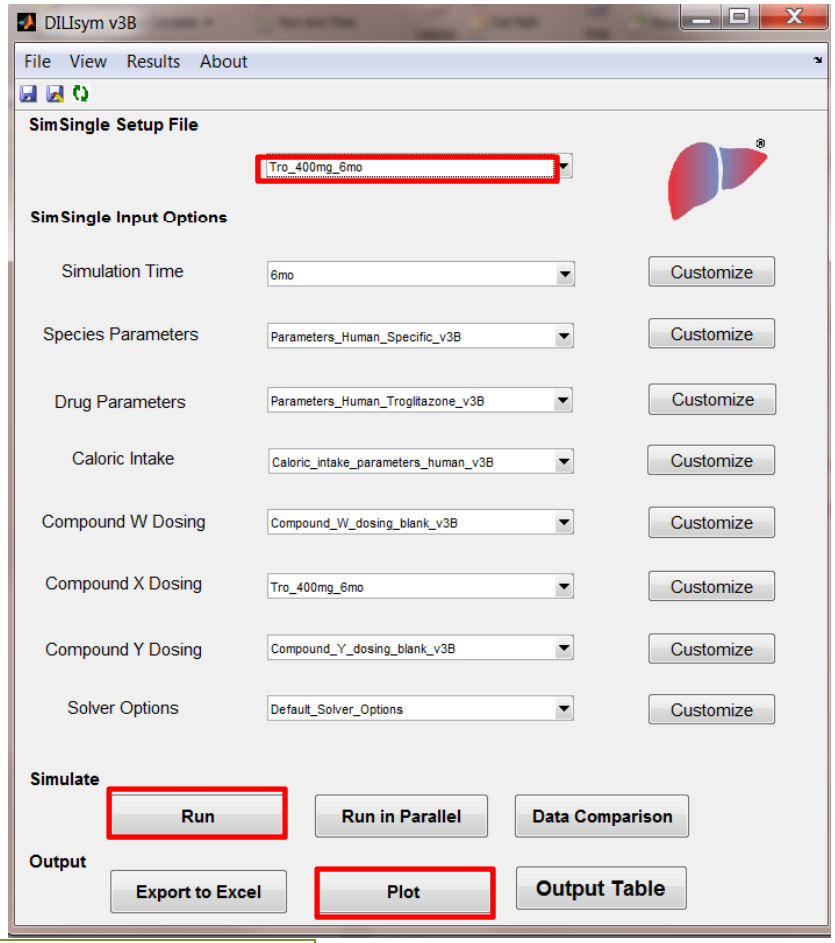
RATS (PO)

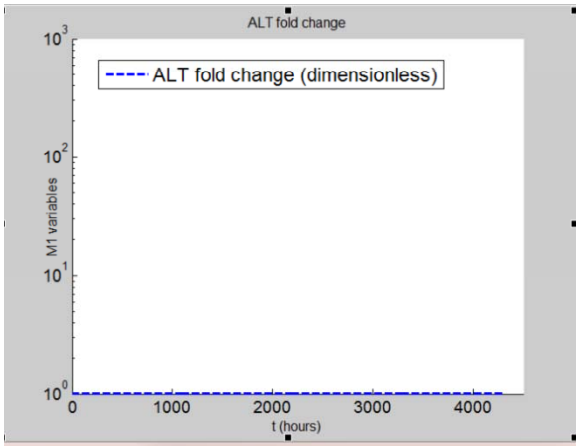
Preclinical and Clinical Data and Simulation Results

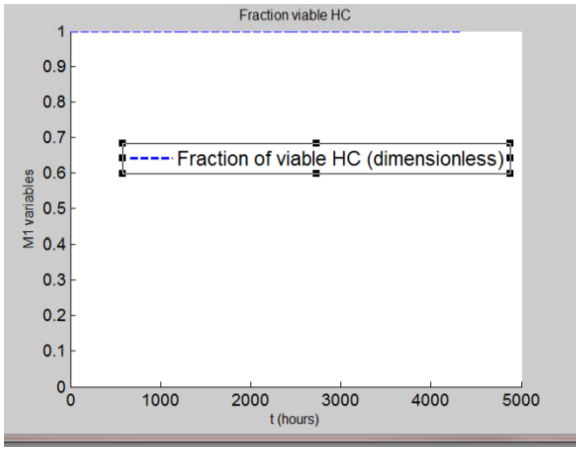
Simulating Troglitazone-Mediated Hepatotoxicity in Baseline Human

HUMANS

Troglitazone 400mg/day for 6 months





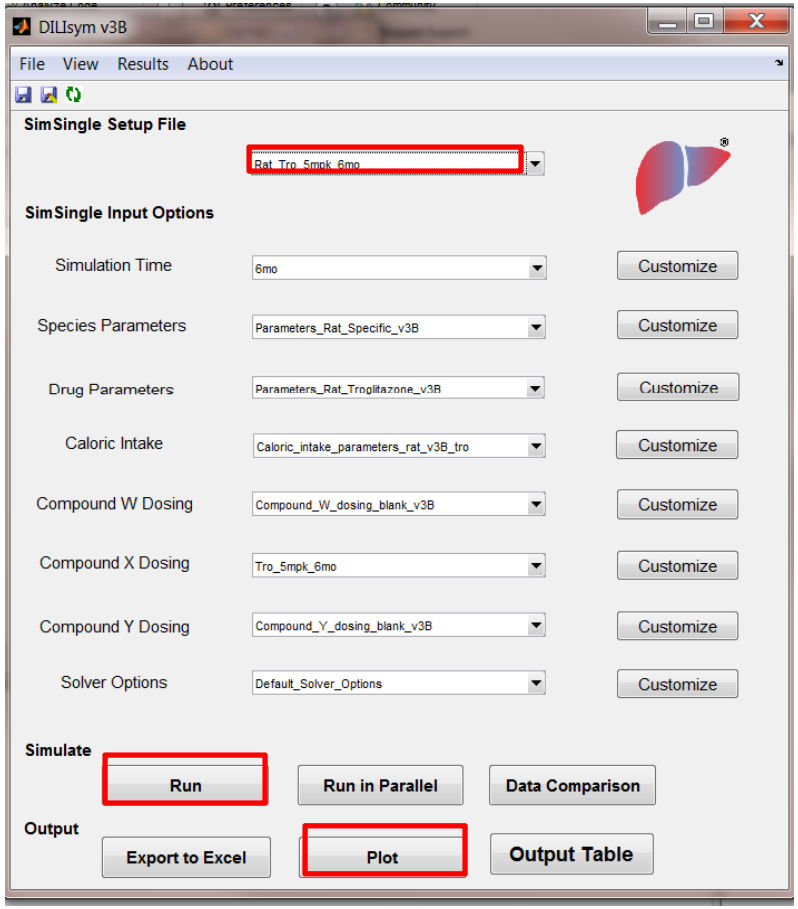


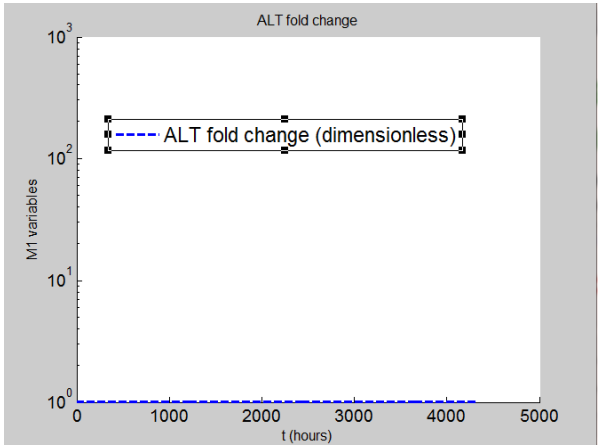
No hepatotoxicity predicted in baseline human

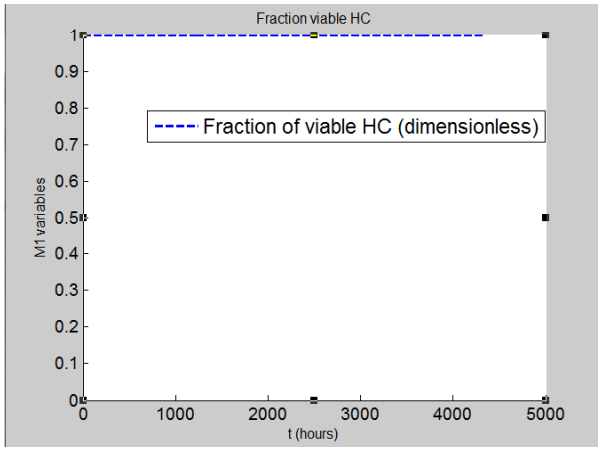
Simulating Troglitazone-Mediated Hepatotoxicity in Baseline Rat

RATS

Troglitazone 5mg/kg/day for 6 months

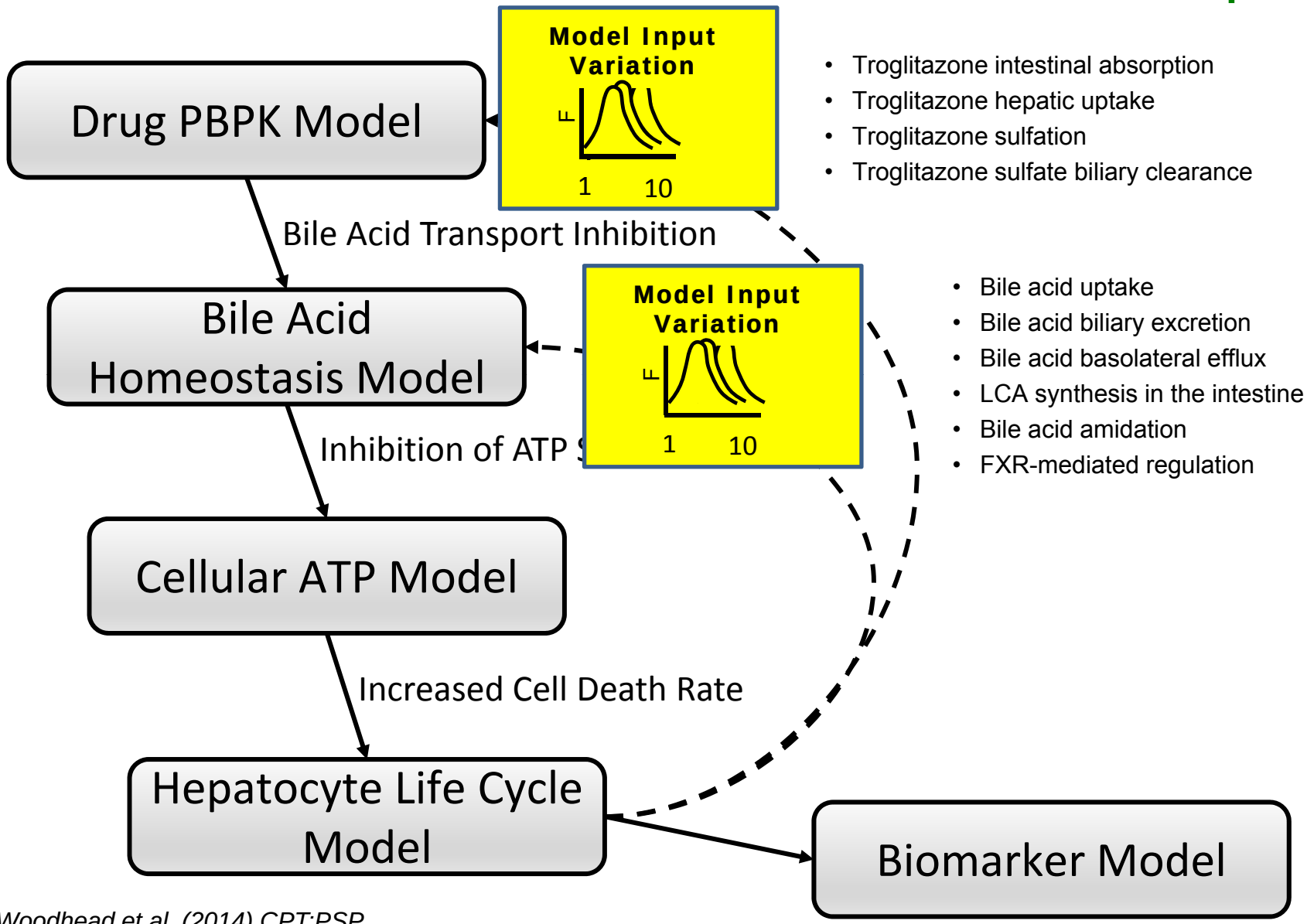






No hepatotoxicity predicted in baseline rat

Construction of Human and Rat SimPops™



Woodhead et al. (2014) CPT:PSP
Yang et al. CPT (in press)

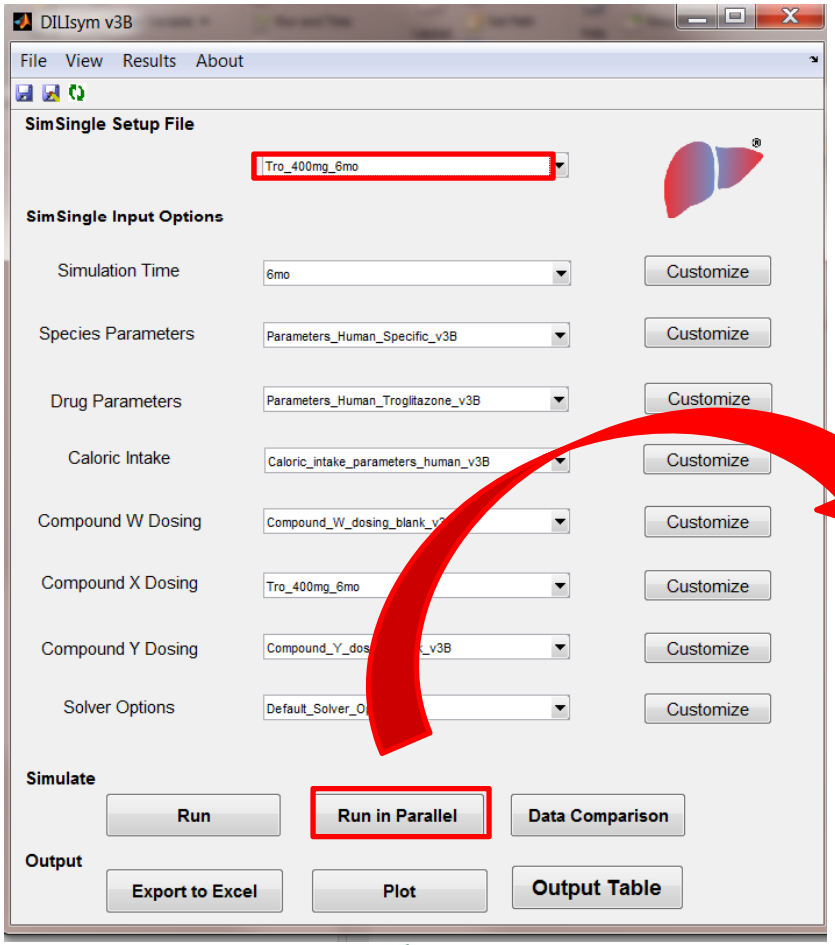
Simulating Troglitazone-Mediated Hepatotoxicity in Human SimPops™

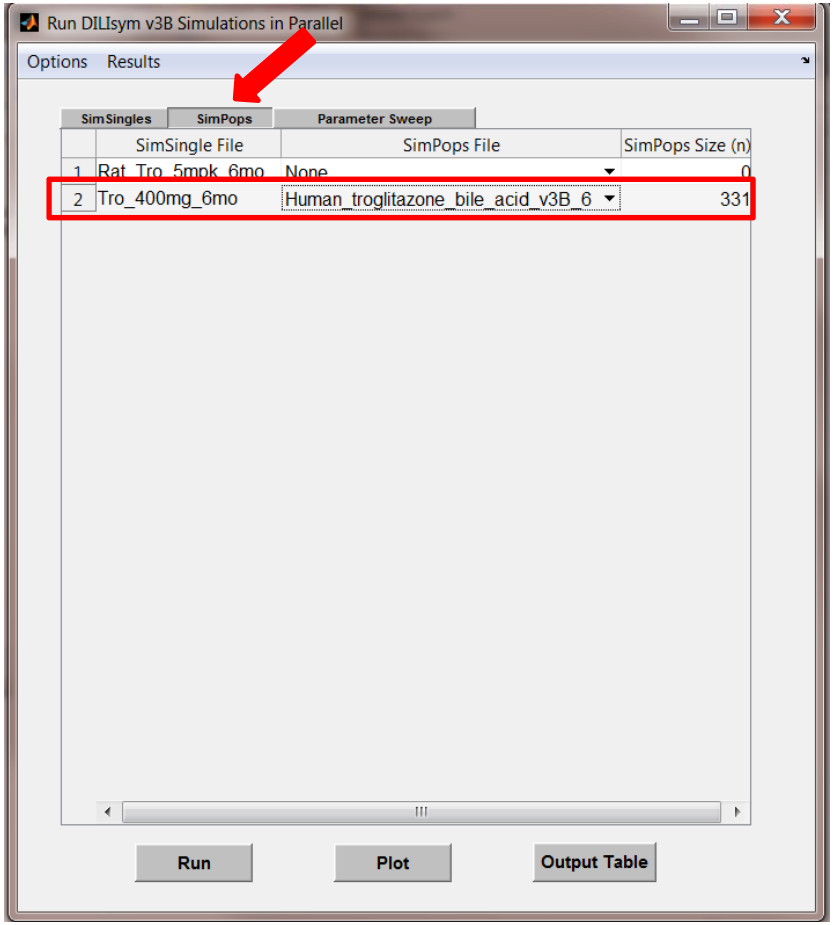
HUMANS

Troglitazone 400mg/day for 6 months

SimPops™

Human_troglitazone_bile_acid_ v3B_6



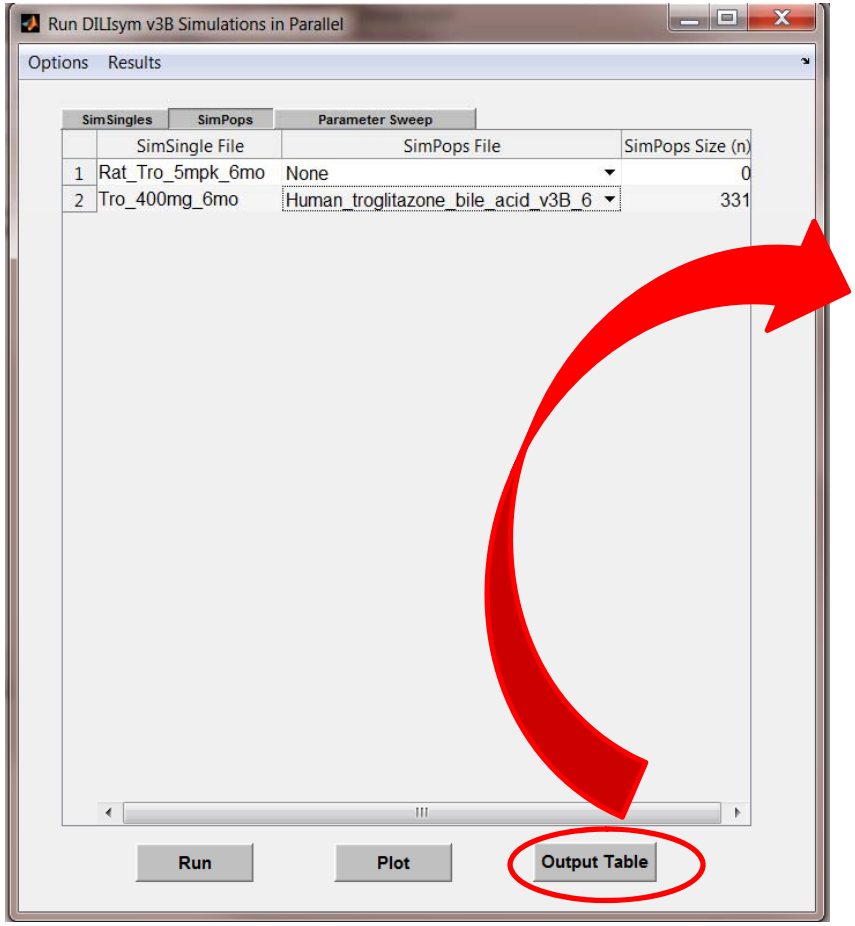


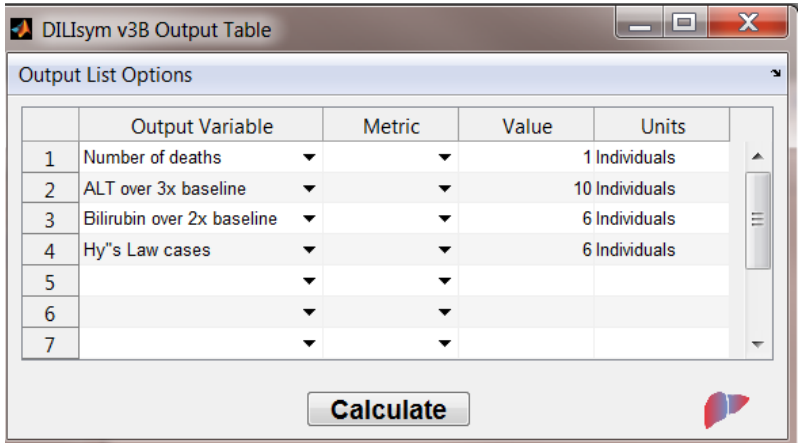
Exploring Troglitazone-Mediated Hepatotoxicity in Human SimPops™

Load SimPops™ Results

Tro_400mg_6mo_v3B6

HUMANS





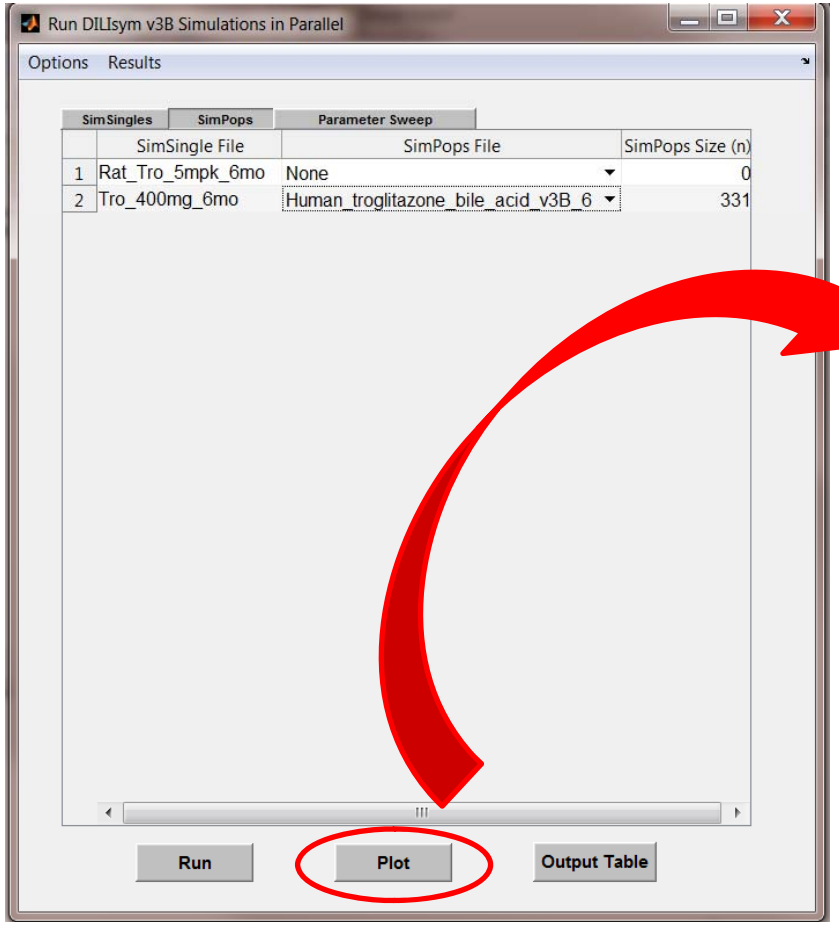
Troglitazone 400mg/day for 6 months

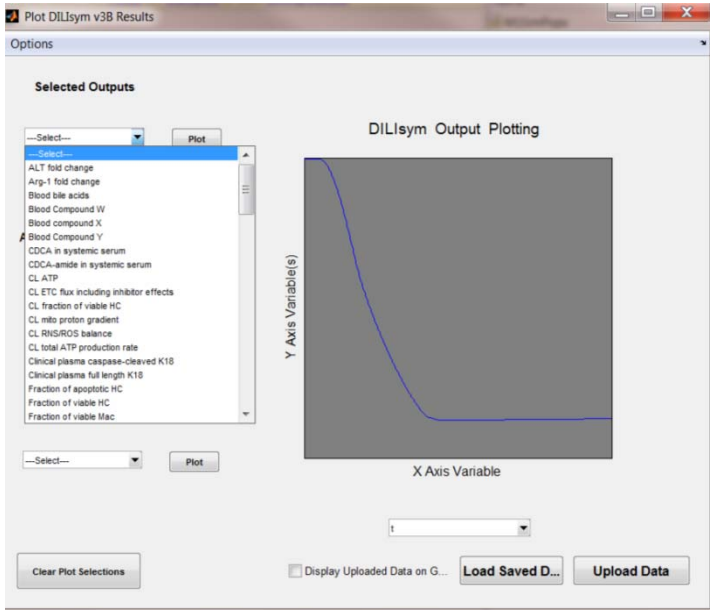
Output	Incidence
Number of deaths	1 / 331 (0.3%)
ALT elevations > 3X	10 / 331 (3.0%)
Bilirubin elevations > 2X	6 / 331 (1.8 %)
Hy's Law cases	6 / 331 (1.8 %)

Exploring Troglitazone-Mediated Hepatotoxicity in Human SimPops™

Load SimPops™ Results

Tro_400mg_6mo_v3B6





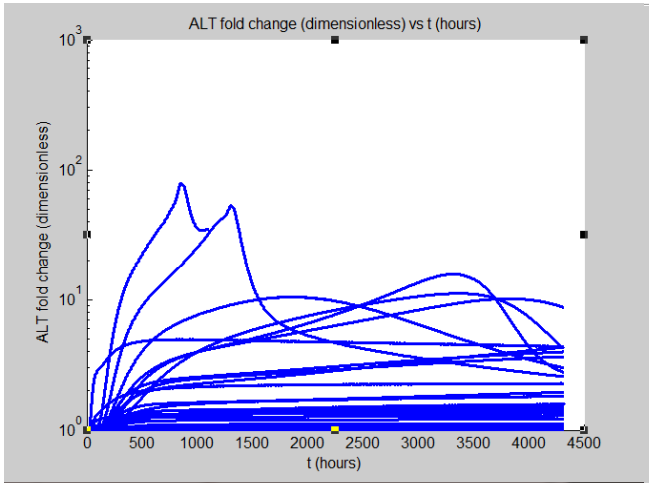
Plot time course of

- ALT fold change
- Plasma bilirubin
- Fraction of viable HC

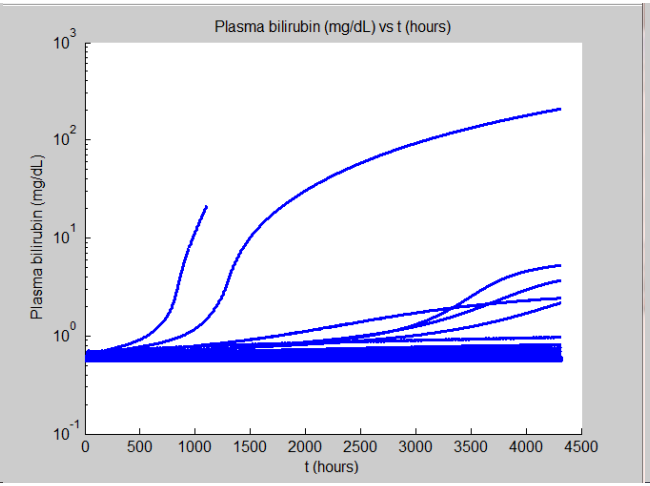
HUMANS

Exploring Troglitazone-Mediated Hepatotoxicity in Human SimPops™

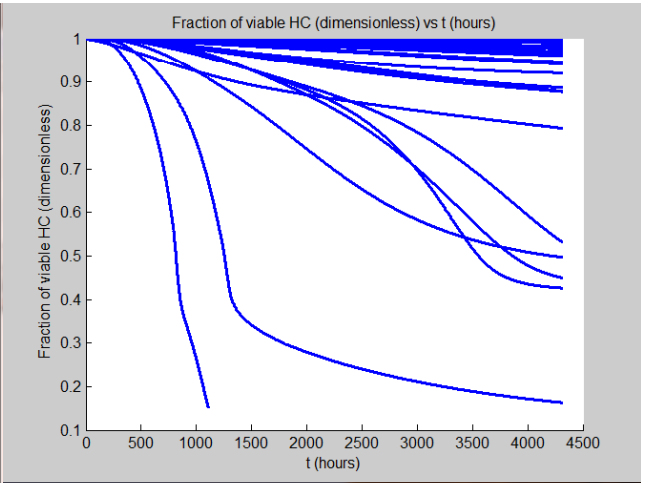
ALT fold change



Plasma bilirubin



Fraction viable HC



- Simulations predict delayed ALT peak in a subset of individuals
  - ALT elevation > 3X in 3% of the population (10/331)
  - Time to peak: 118 ± 61 days
- Bilirubin increase follows ALT elevations
  - Bilirubin elevation > 2X in 1.8% of the population (6/331)
- Significant loss of viable hepatocytes predicted in a subset of individuals
  - One individual lost >85% viable liver mass and identified as dead

HUMANS

Human SimPops™ Results Summary

	Simulations[a]			Clinical Trials[2,3]	
	TGZ 200 mg (n=331)	TGZ 400 mg (n=331)	TGZ 600 mg (n=331)	TGZ 200 – 600 mg (n=2510)	Placebo (n=475)
ALT > 3X ULN (%)[b]	0.3	3.0	5.1	1.9	0.6
ALT > 5X ULN (%)[b]	0.3	1.8	4.2	1.7	N/A
ALT > 8X ULN (%)[b]	0.3	1.8	3.6	0.9	0
ALT > 30X ULN (%)[b]	0	0.6	0.9	0.2	0
Time to peak ALT (Days)[c]	180[d]	118 ± 61	111 ± 61	147 ± 86	N/A
Total Bilirubin > 2X (%)[e]	0.3	1.8	3.6	N/A	N/A
Hy's Law cases (%)	0.3	1.8	3.6	N/A	N/A
Jaundice (%)	N/A	N/A	N/A	0.08	0

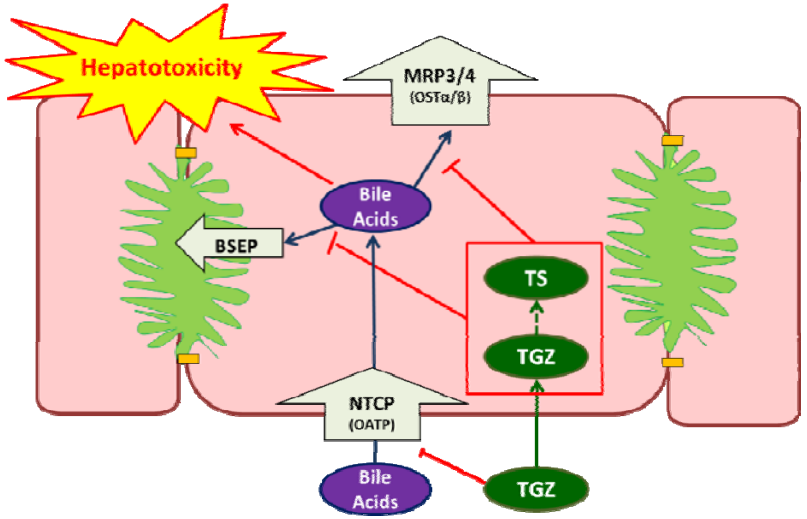
[a] Each dose level was simulated for 6 months.
[b] Upper limit of normal (ULN) was 34 U/L in the clinical trials. In the human SimPops, ULN was 30 U/L because all the individuals had the same baseline ALT (30 U/L) before troglitazone administration.
[c] Mean ± S.D.
[d] S.D. was not calculated because only one individual showed ALT elevation > 3X ULN
[e] Baseline serum total bilirubin in human SimPops was 0.55 mg/dL.
N/A, not available.

Yang et al. CPT (in press)

HUMANS

Clinical Data and Simulation Results

What is the Contribution of Troglitazone Sulfate to the Hepatotoxicity?



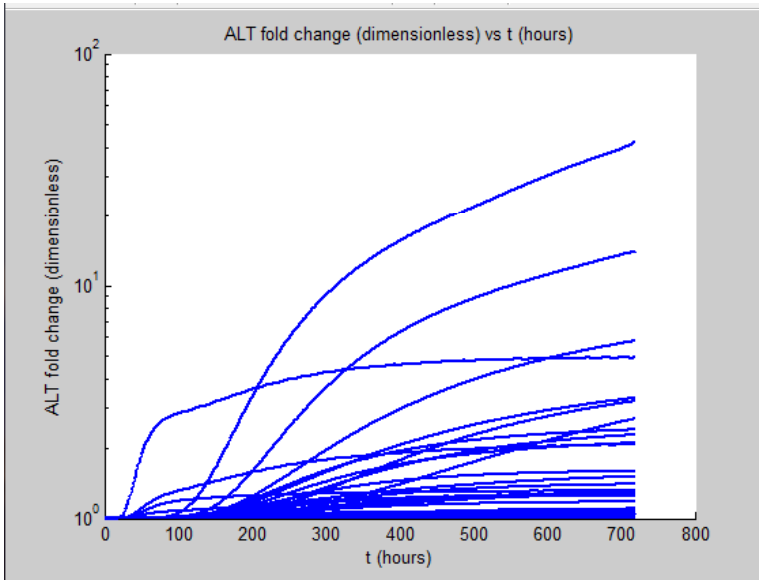
- Troglitazone sulfate is a more potent BSEP inhibitor compared to troglitazone
- Systemic/hepatic exposure to troglitazone sulfate is greater than troglitazone

DILIsym® Parameter Name	DILIsym® Parameter Description	DILIsym® Parameter Input	DILIsym® Parameter Input
Ki_BSEP_CompX_MetB	Competitive Ki for BSEP; Compound X Metabolite B	1.20E-04 mg/mL	1.00E+10 mg/mL
Ki_NTCP_CompX_MetB	Competitive Ki for NTCP; Compound X Metabolite B	1.46E-04 mg/mL (human); 1.02E-03 mg/mL (rat)	1.00E+10 mg/mL
Ki_noncom_baso_CompX_MetB	Noncompetitive Ki for basolateral efflux; Compound X Metabolite B	4.17E-03 mg/mL	1.00E+10 mg/mL

Troglitazone Sulfate is an Important Contributor for Hepatotoxicity

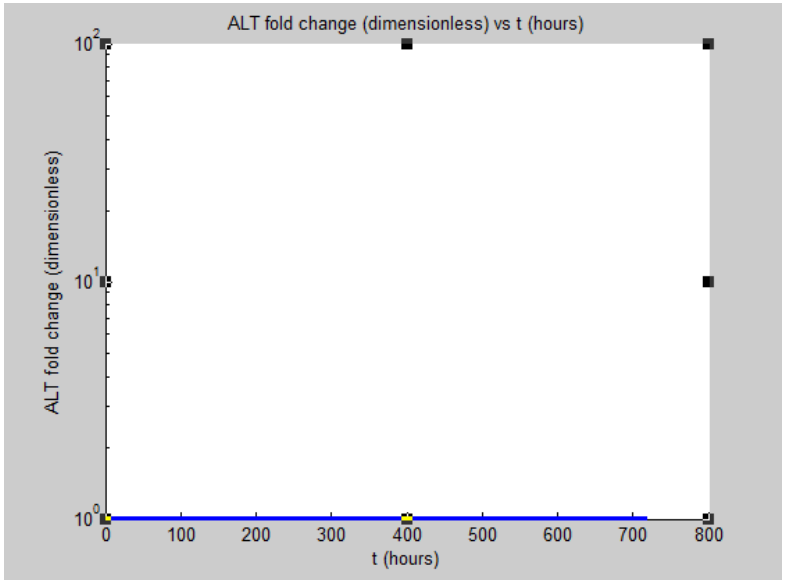
HUMANS

Troglitazone 400mg/day for 1 months



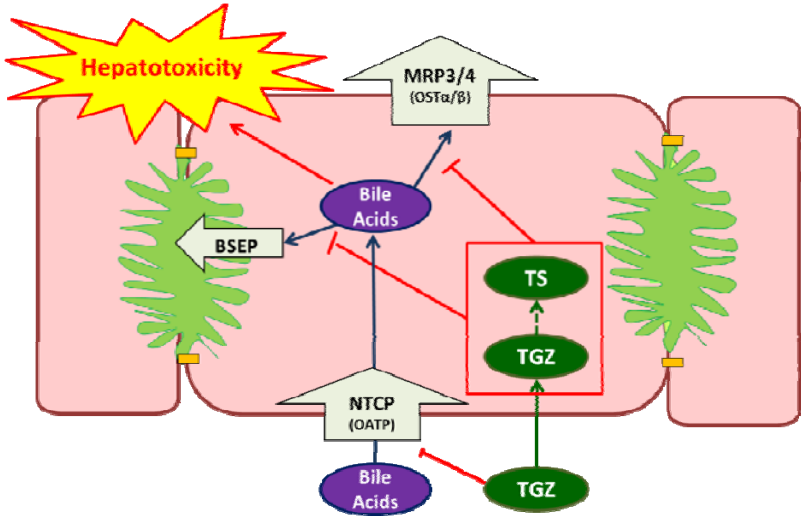
Output	Incidence
Number of deaths	0 / 331 (0%)
ALT elevations > 3X	6 / 331 (1.8%)
Bilirubin elevations > 2X	1 / 331 (0.3%)
Hy's Law cases	1 / 331 (0.3%)

Troglitazone 400mg/day for 1 months – No troglitazone sulfate effects



Output	Incidence
Number of deaths	0 / 331 (0%)
ALT elevations > 3X	0 / 331 (0%)
Bilirubin elevations > 2X	0 / 331 (0%)
Hy's Law cases	0 / 331 (0%)

What is the Contribution of Basolateral Efflux Inhibition to the Hepatotoxicity?



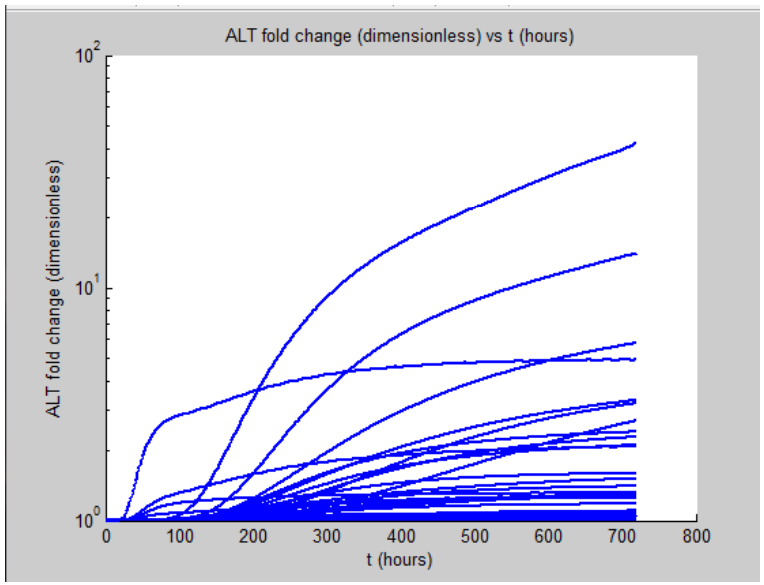
- Bile acids are predominantly excreted into bile via BSEP
- Basolateral efflux transporters serve as compenstory pathways for bile acid excretion when biliary excretion is impaired

DILIsym® Parameter Name	DILIsym® Parameter Description	DILIsym® Parameter Input	DILIsym® Parameter Input
Ki_noncom_baso_CompX	Noncompetitive Ki for basolateral efflux; parent Compound X	9.27E-03 mg/mL	1.00E+10 mg/mL
Ki_noncom_baso_CompX_MetB	Noncompetitive Ki for basolateral efflux; Compound X Metabolite B	4.17E-03 mg/mL	1.00E+10 mg/mL

Inhibition Data for Multiple Transporters are Important for Predicting Hepatotoxicity

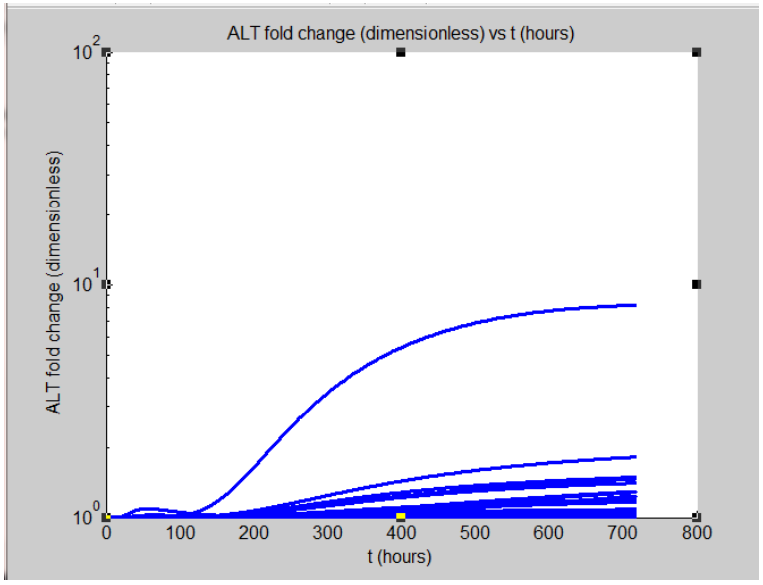
HUMANS

Troglitazone 400mg/day for 1 month



Output	Incidence
Number of deaths	0 / 331 (0%)
ALT elevations > 3X	6 / 331 (1.8%)
Bilirubin elevations > 2X	1 / 331 (0.3%)
Hy's Law cases	1 / 331 (0.3%)

Troglitazone 400mg/day for 1 months – No MRP4 inhibition



Output	Incidence
Number of deaths	0 / 331 (0%)
ALT elevations > 3X	1 / 331 (0.3%)
Bilirubin elevations > 2X	0 / 331 (0%)
Hy's Law cases	0 / 331 (0%)

Exploring Troglitazone-Mediated Hepatotoxicity in Rat SimPops™

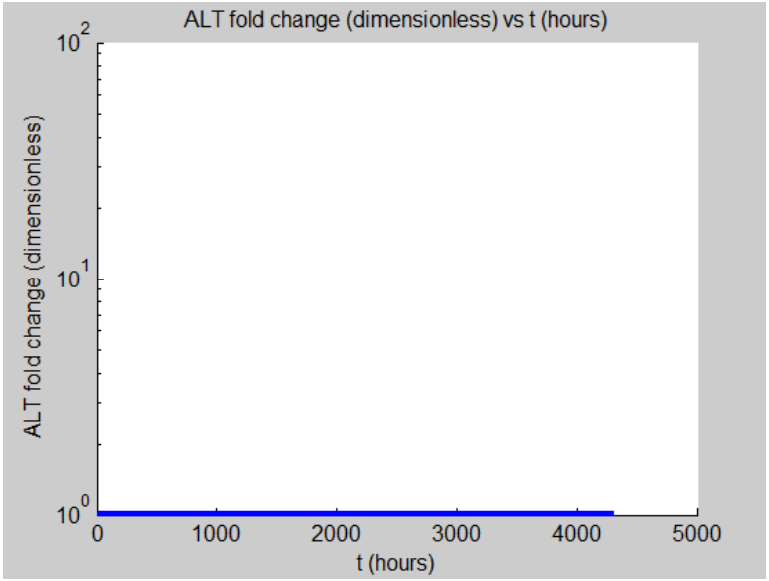
Troglitazone 5 mg/kg/day for 6 months

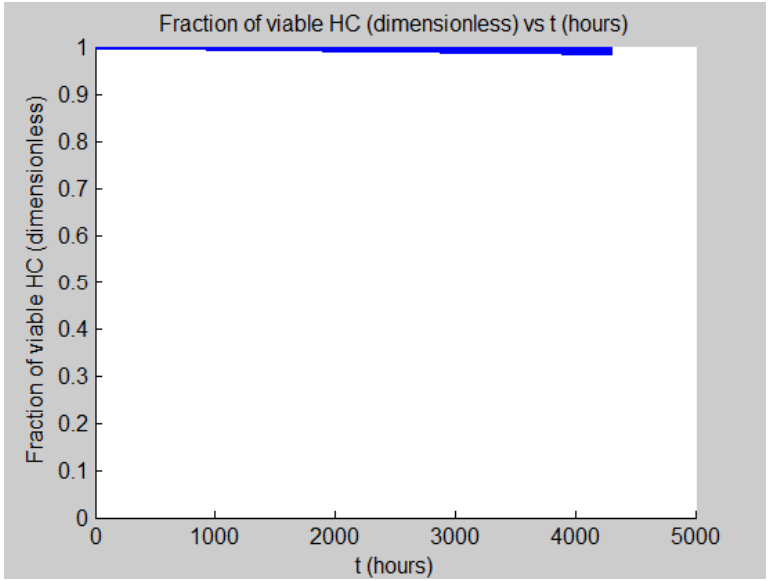
Rat_troglitazone_bile_acid_v3B_8

Output	Incidence
Number of deaths	0 / 191 (0%)
ALT elevations > 3X	0 / 191 (0%)
Bilirubin elevations > 2X	0 / 191 (0%)
Hy's Law cases	0 / 191 (0%)

No hepatotoxicity predicted in rat SimPops™

RATS





Summary

- DILIsym® data inputs for bile acid transport inhibition can be measured from *in vitro* studies
  - Recommended experimental systems include membrane vesicles (efflux transporters), transfected cell lines or primary hepatocytes (uptake transporters)
  - Determination of Ki and type of inhibition recommended
  - Inhibition data for multiple bile acid transporters using both parent and major stable metabolites would provide more reliable predictions
- Troglitazone-mediated hepatotoxicity was simulated using DILIsym® based on *in vitro* bile acid transport inhibition data
  - Incidence and delayed presentation of troglitazone hepatotoxicity were predicted in the simulated human population
    - Inhibitory effects of troglitazone sulfate on bile acid transporters were critical to hepatotoxicity
    - Inhibition data for multiple transporters were critical
  - TGZ was not hepatotoxic in the simulated rat population

DILIsym® Training Agenda – September 11, 2014

- 8:30 AM – Introduction
  - Training session goals
  - DILIsym® v3B overview and highlights
  - Model architecture notes
- 8:45 AM – Modeling troglitazone with DILIsym®
- 9:45 AM – Break
- 10:00 AM – Modeling entacapone and tolcapone with MITOsym® and DILIsym®
- 11:00 AM – Modeling compounds that disturb reactive oxygen species balance
- 11:30 AM – Lunch
- 12:30 PM – Discussion Topics
  - Data needs and use for PBPK modeling within DILIsym®
  - Free vs. total drug concentrations as determinants of toxicity mechanisms
  - DILIsym® equation design
  - *Biomarker design within DILIsym® (if time permits)*
  - *Timing of injury and injury progression within DILIsym® (if time permits)*
- 2:45 PM – Open discussion and wrap up
- 3:00 PM – Training concludes
  - DILI-sim modeling team is available for questions

Predicting *in vivo* DILI Risk Based on Dual Toxicity Mechanisms: A Case Study with Entacapone and Tolcapone

- Introduction
  - Entacapone and Tolcapone display hepatotoxic effects on the mitochondrial proton gradient and on hepatic bile acid transporters
- Developing models of entacapone and tolcapone that incorporate dual toxicity mechanisms
  - Simulate uncoupling effects in MITOsym® and translate parameter values to DILIsym®
  - Gather data inputs for bile acid transport inhibition and translate data to DILIsym® parameter values
  - Construction of PBPK models
- Simulating hepatotoxicity profiles for entacapone and tolcapone using DILIsym®
  - Simulate and compare responses to entacapone and tolcapone in baseline human
  - Simulate entacapone and tolcapone within the human SimPops™ and compare hepatotoxicity profiles

Entacapone and Tolcapone: Similar Mechanistic Effects but Differences in Clinical Hepatotoxicity

- Entacapone and tolcapone represent a "clean/toxic" compound pair
  - Similar structure and pharmacologic mechanism
  - No hepatotoxicity reported for patients taking entacapone
  - Clinical hepatotoxicity observed with tolcapone
- Similar mechanistic hepatotoxic effects for entacapone and tolcapone
  - Both compounds uncouple the mitochondria proton gradient
  - Modest BSEP inhibition with entacapone (IC_{50}=55.6 µM, Morgan 2013) and tolcapone (IC_{50}=36.6 µM, Morgan 2013)
- Can DILIsym® recapture the differences in hepatotoxicity observed clinically based on mechanistic information?

HUMANS

ENTACAPONE

Parameter	RCTs		EXT		Placebo	
	NDA n=406	Overall n=603	NDA n=325	Overall n=738	NDA n=296	Overall n=400
Total bilirubin	0.3%	0.2%	0	0.2%	0	0
AST	0.3%	0.3%	0	0.2%	0.7%	0.3%
ALT	0.5%	0.5%	0	0.2%	0.8%	0.6%
GGT	0	0.4%	0.3%	0.3%	0.4%	0*
Alkaline Phosphatase	0	0	0	0	0.4%	0*

FDA Comtan safety document

TOLCAPONE

Adverse Event	*Placebo*	*100 mg*	*200 mg*
PHASE III CONTROLLED TRIALS	(n=292)	(n=294)	(n=293)
High SGPT (ALAT)			
≥2x ULN			
>3x ULN	0	3 (1%)	8 (3%)
>5x ULN	0	2 (0.7%)	3 (1%)
>8x ULN	0	1 (0.3%)	1 (0.3%)
High SGOT (ASAT)			
≥2x ULN			
>3x ULN	0	4 (1%)	6 (2%)
>5x ULN	0	2 (0.7%)	3 (1%)
>8x ULN	0	0	2 (0.7%)
High alkaline phosphatase	2 (1%)	0	1 (0.3%)

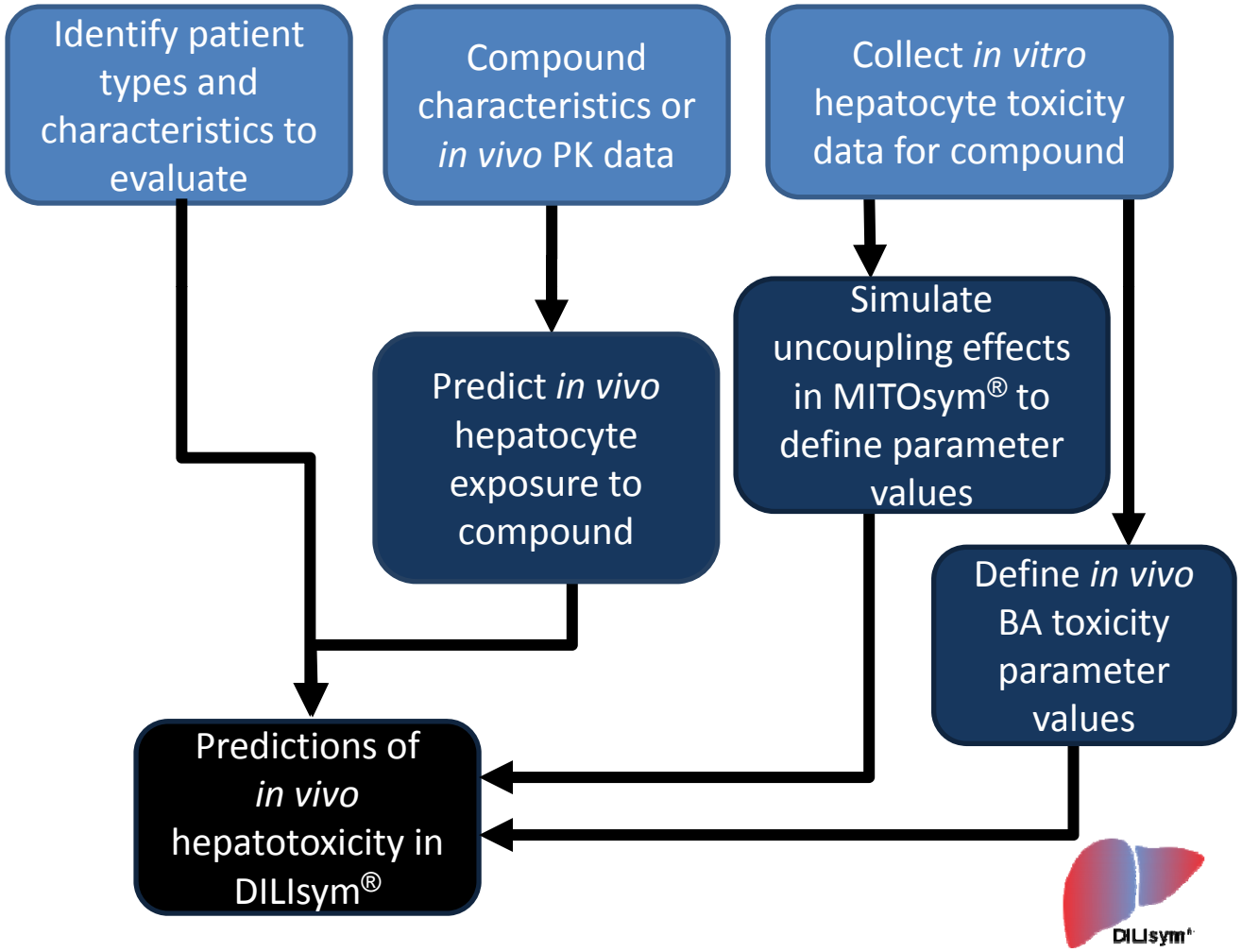
Tasmar FDA filing documents

Workflow for Modeling Entacapone and Tolcapone with MITOsym® and DILIsym®

Approach: Predict *in vivo* risk based on PK modeling and *in vitro* hepatocyte toxicity data for mitochondrial and BA toxicity mechanisms

Case study: Compare the simulated hepatotoxicity profile between tolcapone and entacapone

Baseline human and SimPops™



Workflow for Modeling Entacapone and Tolcapone with MITOsym® and DILIsym®

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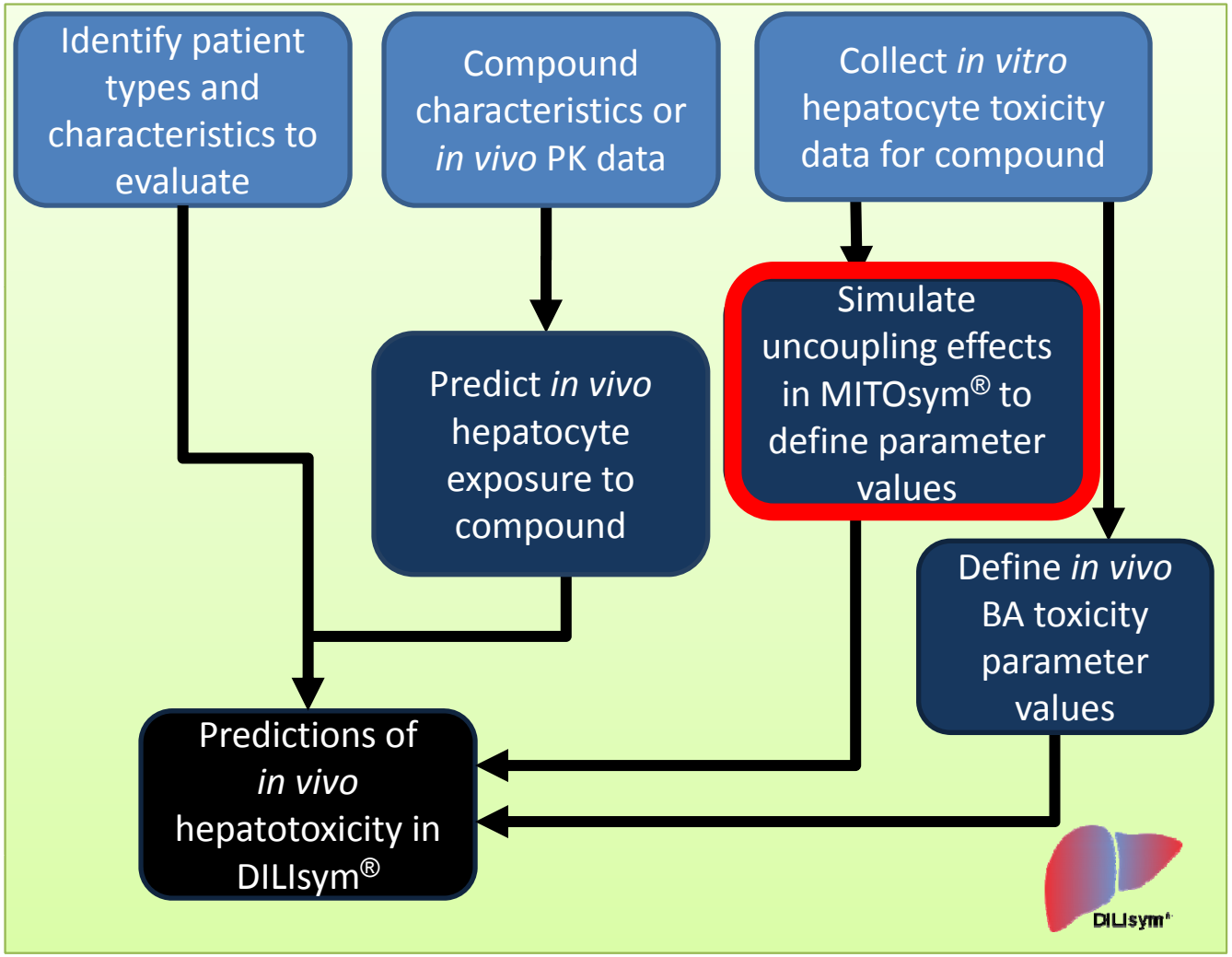
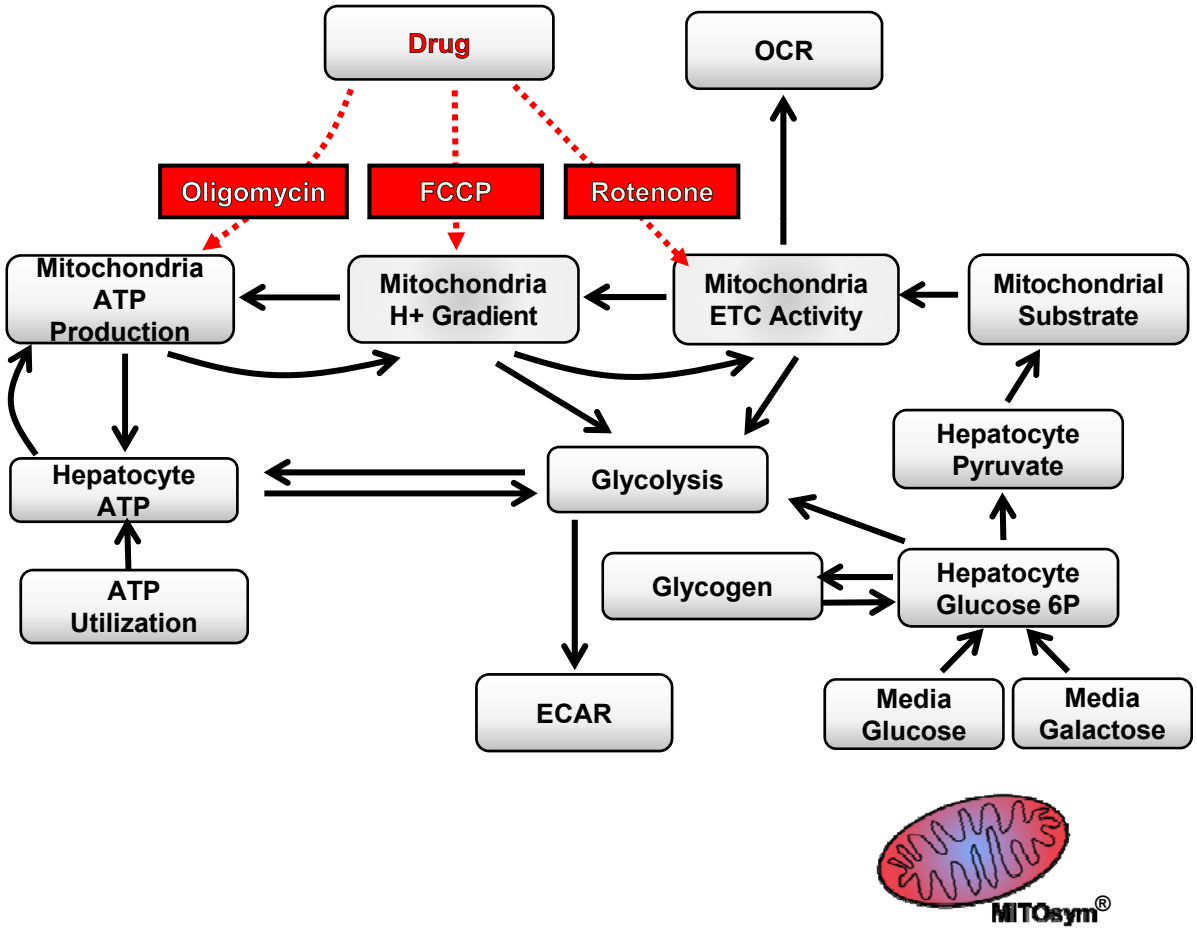


MITOsym® Model Includes Essential Components of Hepatocyte Bioenergetics

- Includes mitochondria ETC activity, proton gradient and ATP production
- Includes respiration (OCR) as a primary model output
  - Also includes ATP, $\Delta\Psi m$, ECAR
- MITOsym® simulates and recapitulates the reported dynamic changes exemplar drugs in HepG2, primary human and rat hepatocytes
- MITOsym® model is designed to provide inputs into the DILIsym® model to predict *in vivo* hepatotoxicity based on *in vitro* data

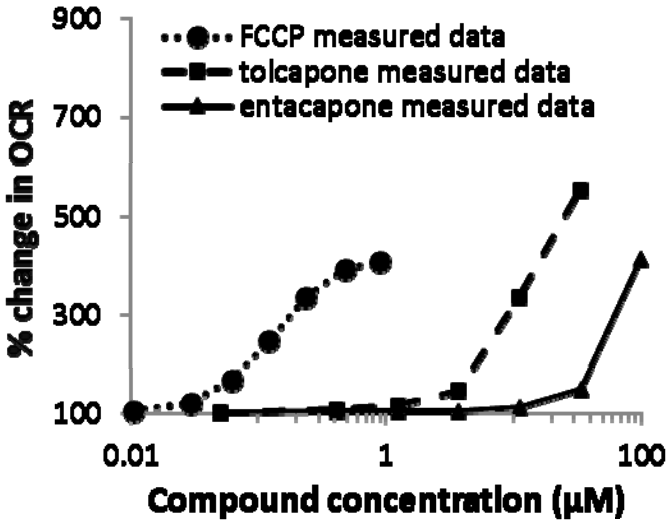


Simulating Uncoupling Effects in MITOsym® to Define Mitochondrial Toxicity Parameter Values

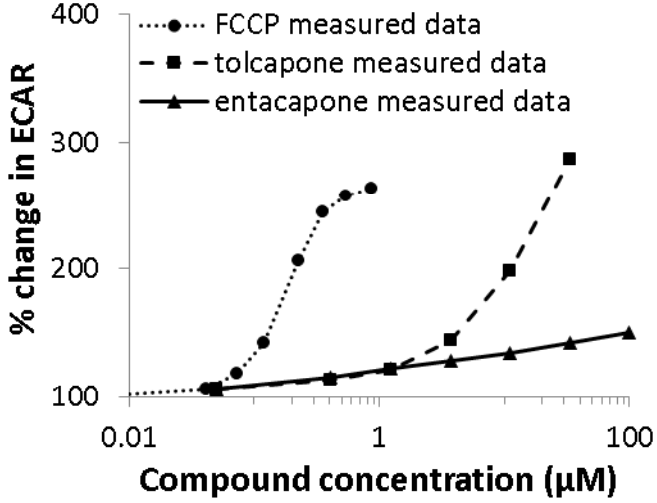
Objective:

- Use MITOsym® model to simulate changes in OCR and ECAR caused by uncoupling
- Determine uncoupling parameter values for entacapone and tolcapone by comparing simulated dose response curves to HepG2 measured data (Nadanaciva 2012)
  - FCCP is a MITOsym® exemplar compound with a strong uncoupling effect
  - Use HepG2 FCCP SimSingle available in MITOsym® as a starting point

HepG2

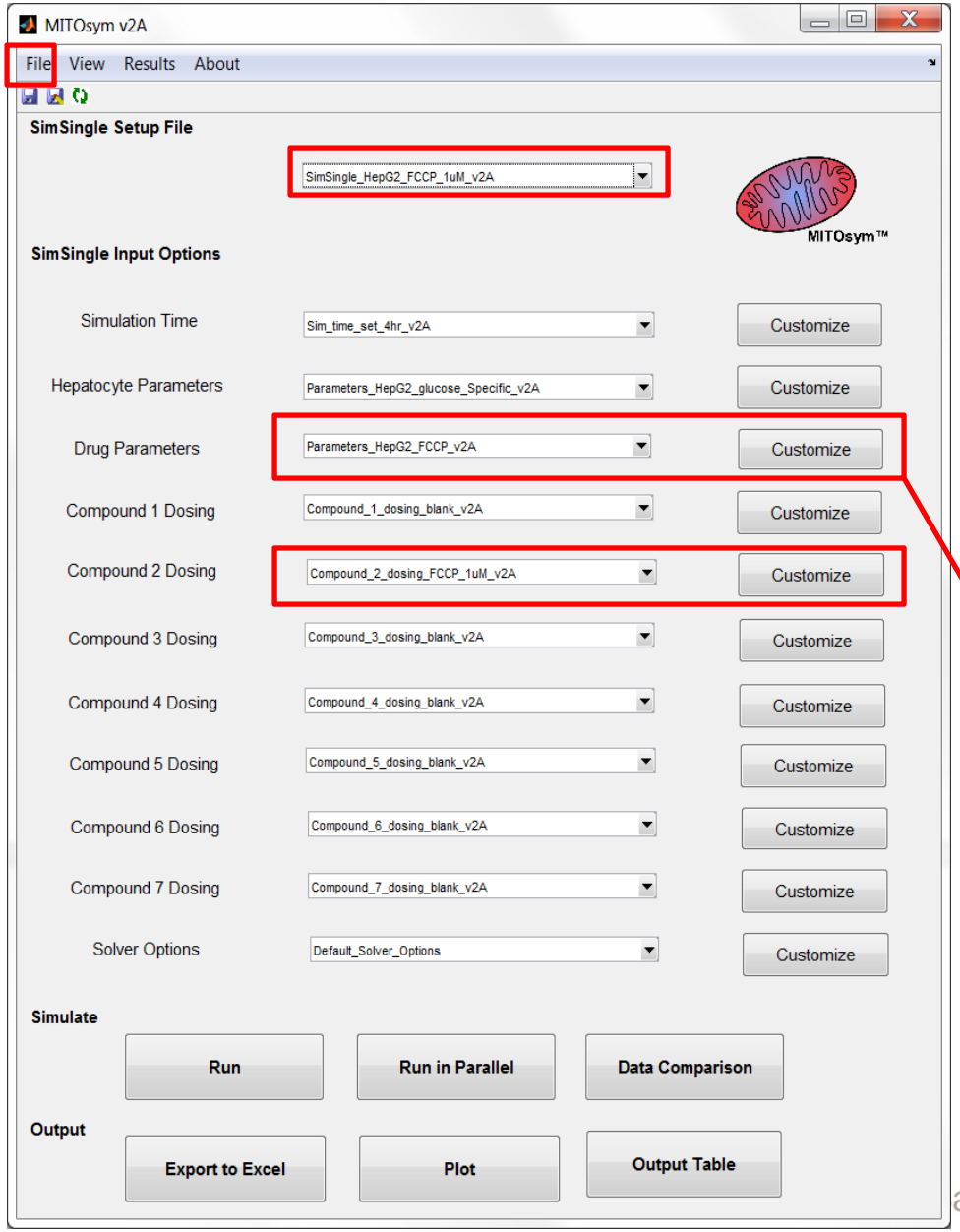


Nadanaciva 2012

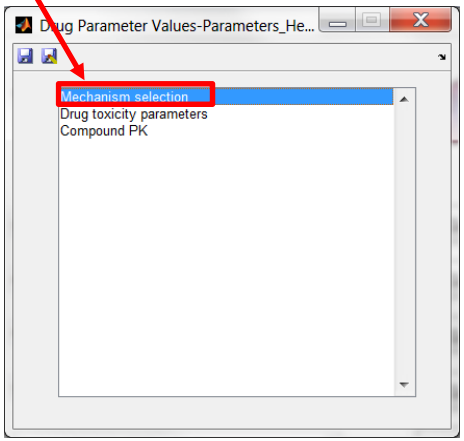


Nadanaciva 2012

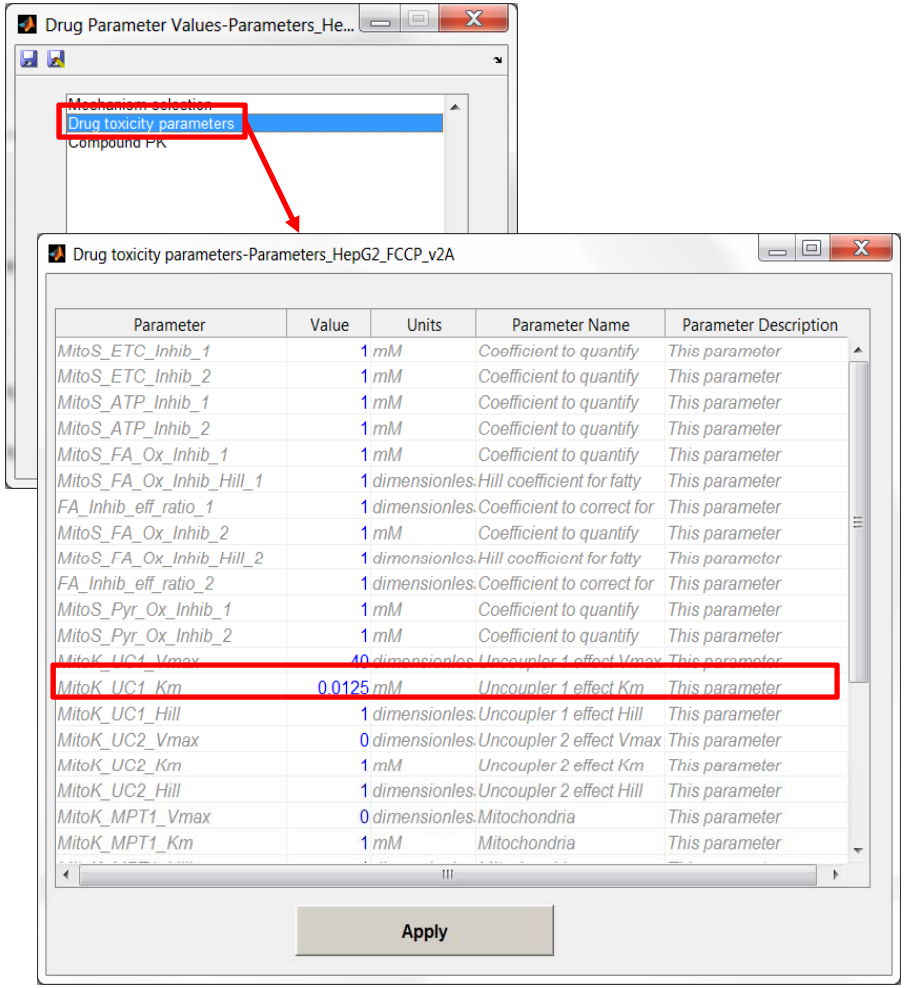
Creating Entacapone SimSingle in MITOsym®



1. Select HepG2 FCCP SimSingle in MITOsym®
2. Save SimSingle as: SimSingle_HepG2_Entacapone_1uM
3. View “Compound 2 Dosing” and save as: Compound_2_dosing_Entacapone
4. Select “Drug Parameters”, rename/save as: Parameters_HepG2_Entacapone
5. View “Mechanism selection”, verify “Mitochondrial uncoupler 1” is selected

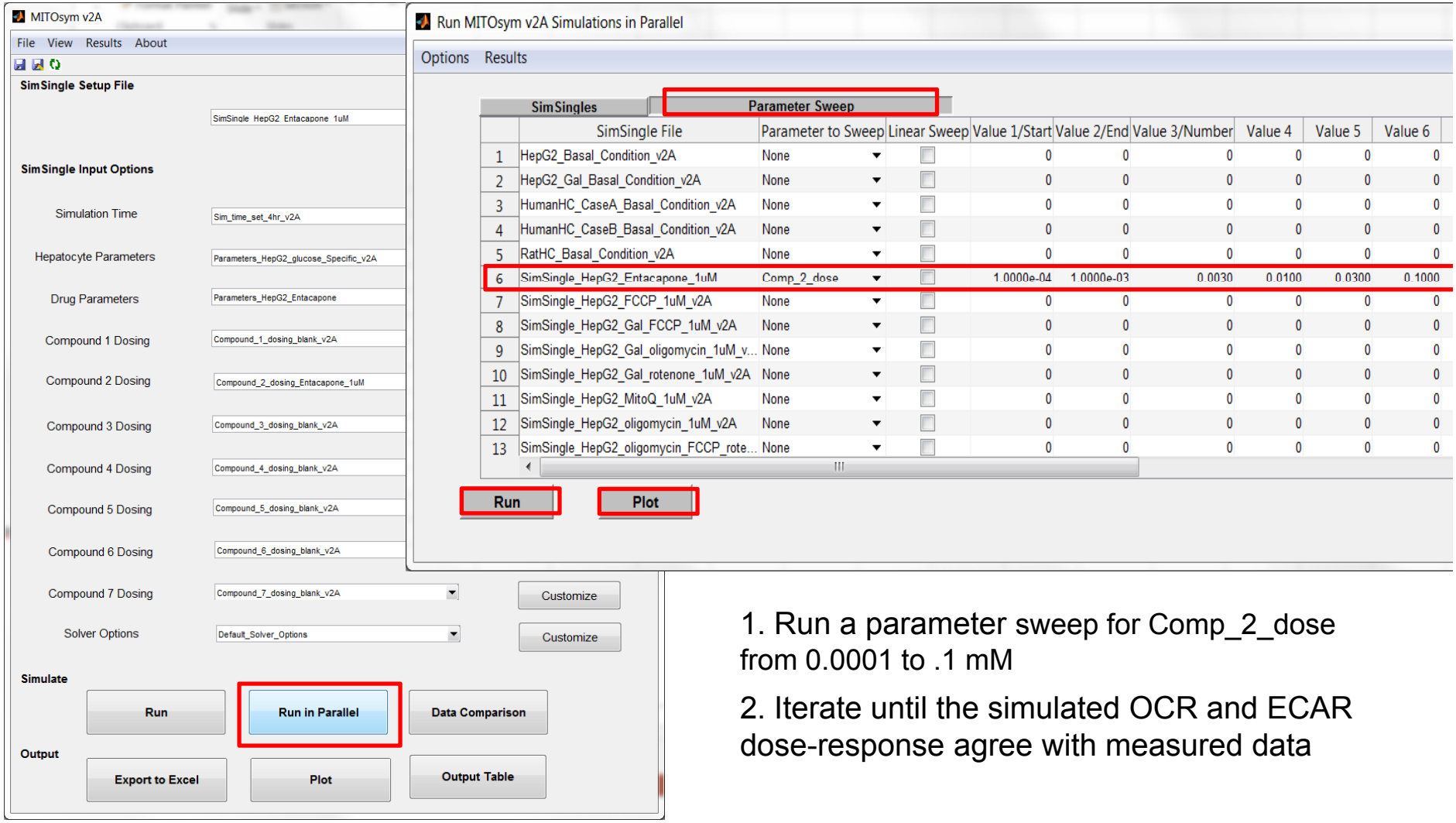


Changing the Uncoupling Drug Toxicity Parameter in MITOsym®



1. View “Drug toxicity parameters”
2. The Km for the effect of Uncoupler 1 is 0.0125 mM for FCCP
3. Based on previous simulation, Km for Tolcapone is about 5X higher than FCCP
4. Entacapone is a much weaker uncoupler than Tolcapone,
   - Try Km ~50x higher than FCCP as a first guess:
     - Change MitoK_UC1_Km to 0.5 mM
     - Apply and Save

Running a Dose Sweep in MITOsym®



1. Run a parameter sweep for Comp_2_dose from 0.0001 to .1 mM

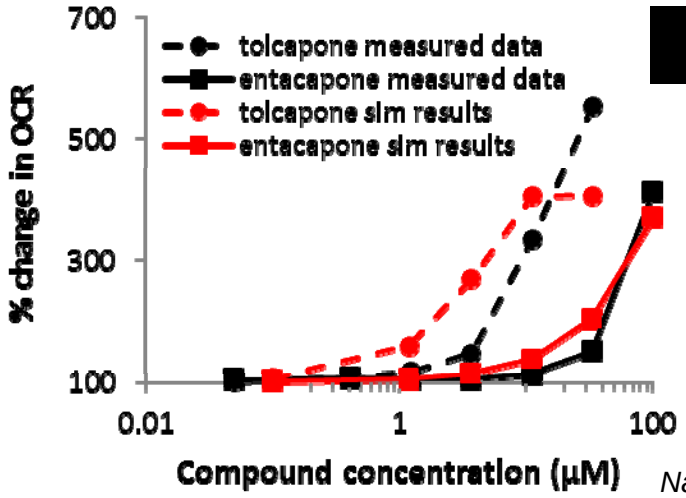
2. Iterate until the simulated OCR and ECAR dose-response agree with measured data

Entacapone and Tolcapone Uncoupler Parameter Values with MITOsym®

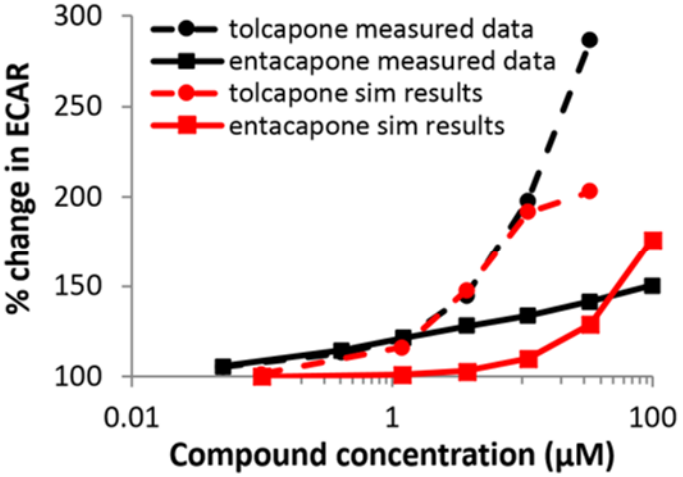
- Used MITOsym® model to simulate OCR and ECAR response to entacapone and tolcapone
  - Good agreement with measured OCR and ECAR data (by design)

- Entacapone is a weaker uncoupler than tolcapone
  - MitoK_UC1_Km parameter value is ~10x greater for entacapone than tolcapone
    - Entacapone Km 1.0
    - Tolcapone Km 0.065

HepG2



Nadanaciva 2012

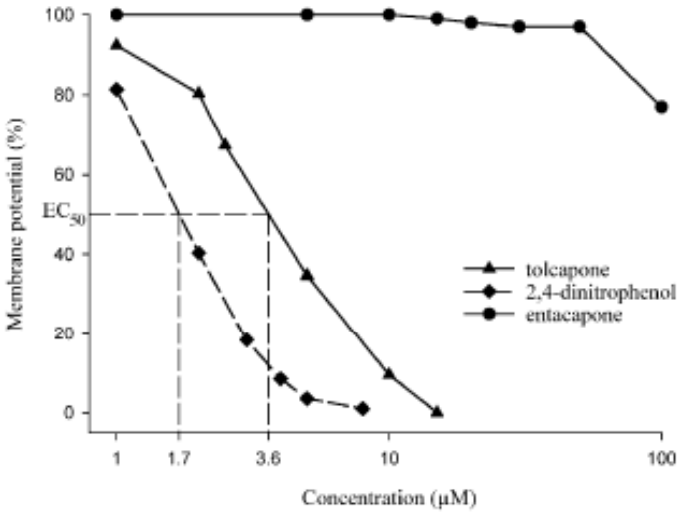


Nadanaciva 2012

Simulated Membrane Potential in Good Agreement with Available Data for Entacapone and Tolcapone

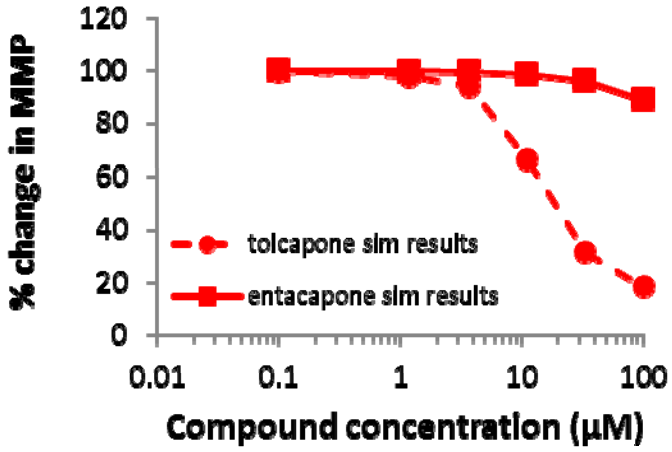
- Haasio directly measured uncoupling in rat liver mitochondria
- Used MITOsym® model to simulate MMP response to tolcapone and entacapone
- Used RLM data (Haasio 2002) as "validation" for tolcapone and entacapone parameters set based on HepG2 OCR and ECAR data (Nada. 2012)

RLM



Haasio 2002

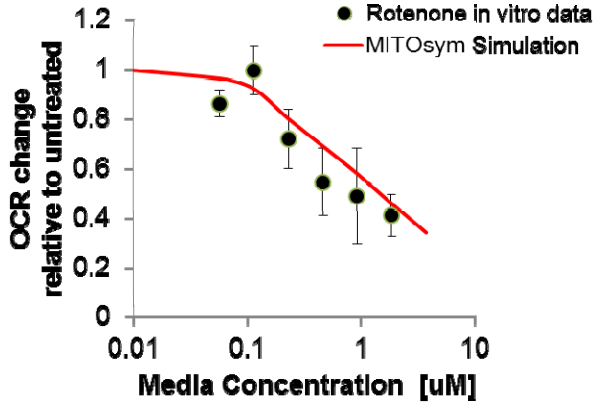
HepG2



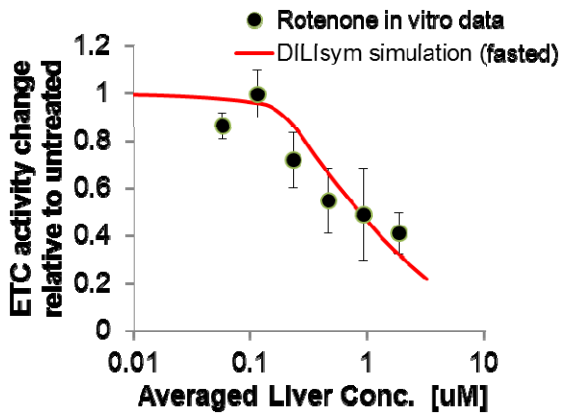
Translating MITOsym® Parameter Values to DILIsym® Parameters using Exemplar Compounds

Exemplar (Rotenone)

Rotenone DR in MITOsym®



Rotenone DR in DILIsym®



Collect *in vitro* hepatocyte toxicity data for compound

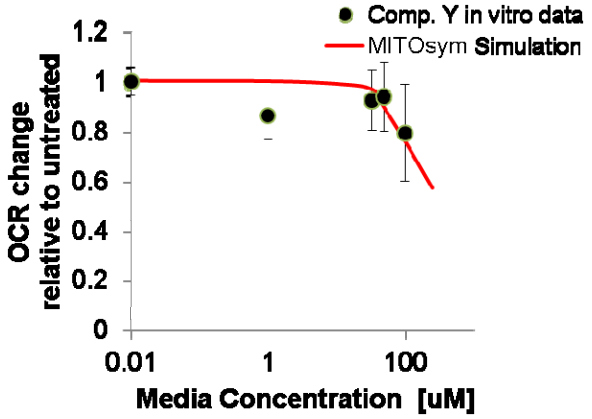
Simulate OCR response in MITOsym® and determine parameter value

Determine DILIsym® parameter value by normalizing vs. exemplar compound

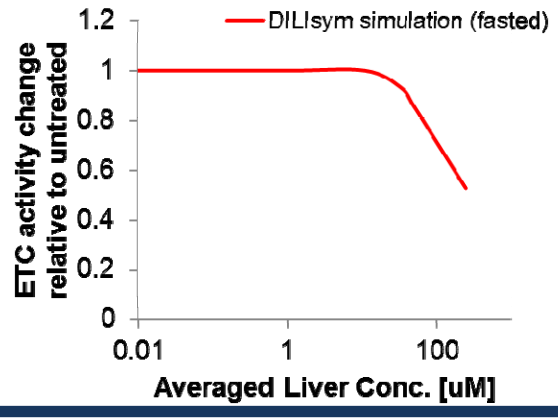
Predictions of *in vivo* hepatotoxicity in DILIsym®

Compound Y

Compound Y DR in MITOsym®



Compound Y DR in DILIsym®



Preclinical Data and Simulation Results

Conversion of Uncoupler Drug Parameter Values Between MITOsym® and DILIsym®

		MITOsym®			DILIsym®		
Drug	**Parameter**	**MITOsym cell type**	**MITOsym mechanism**	**MITOsym parameter value (mM)**	**DILIsym species**	**DILIsym parameter value (mol/mL)**	**Km parameter value relative to FCCP**
Entacapone	MitoK_UC1_Km	HepG2	Uncoupler	1.0	Human	3.20E-06	80x
Tolcapone	MitoK_UC1_Km	HepG2	Uncoupler	0.065	Human	2.08E-07	5.2x
FCCP	MitoK_UC1_Km	HepG2	Uncoupler	0.0125	Human	4.00E-08	1x

- Minor differences between MITOsym® and mitochondria sub-model of DILIsym®
  - Account for differences in mitochondria drug-related parameters
- Parameter values relative to the mitochondria exemplar drugs in MITOsym® are what should be used in DILIsym®

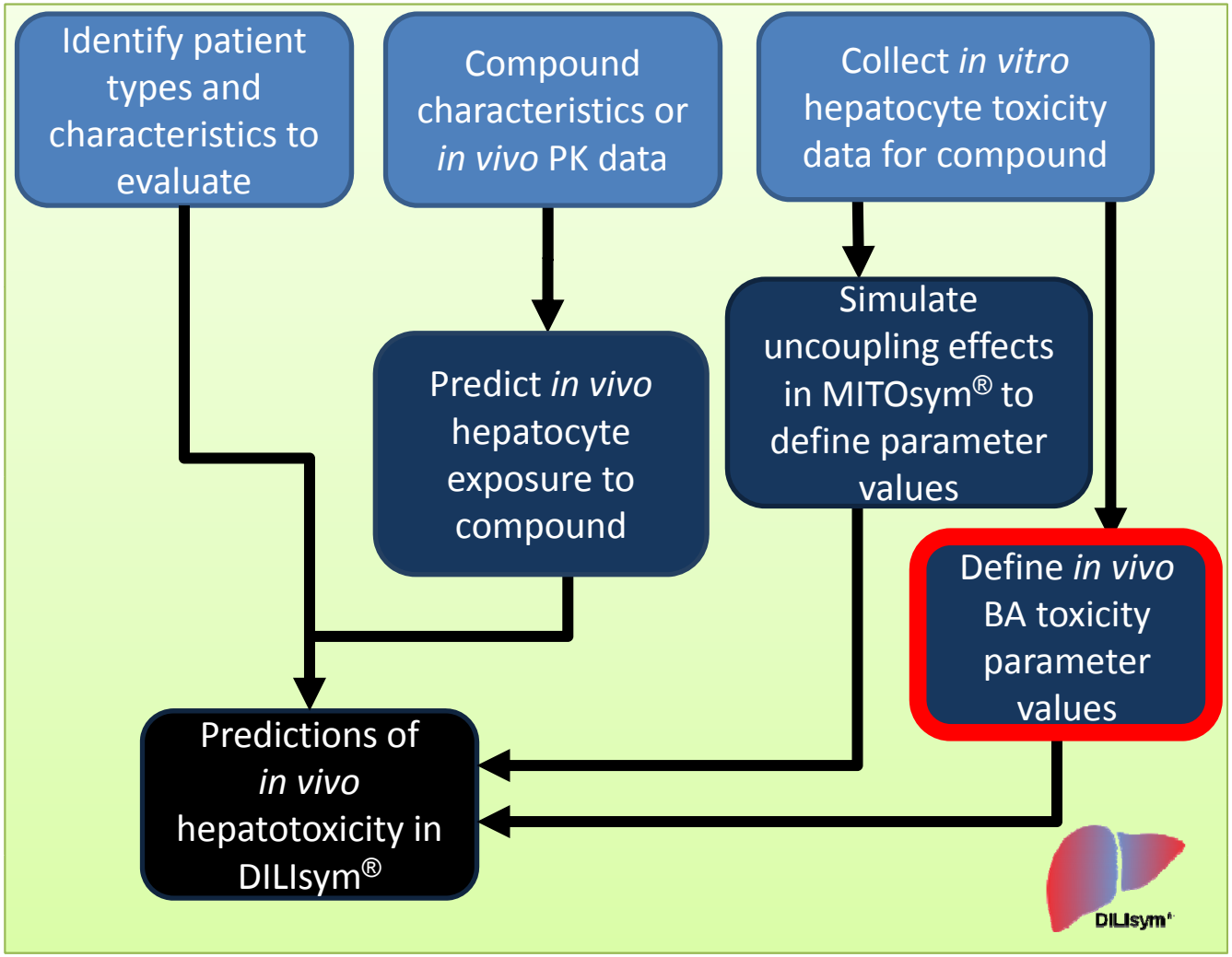
Human DILIsym® Comp Y parameter values = **Ratio of Comp Y to exemplar parameter values in MITOsym®** X **DILIsym® exemplar parameter values**

Workflow for Modeling Entacapone and Tolcapone with MITOsym® and DILIsym®

Approach: Predict *in vivo* risk based on PK modeling and *in vitro* hepatocyte toxicity data for mitochondrial and BA toxicity mechanisms

Case study: Compare the simulated hepatotoxicity profile between tolcapone and entacapone

Baseline human and SimPops™



Gather Data Inputs for Bile Acid Transport Inhibition and Translate to DILIsym® Parameters

- Bile acid transport inhibition constants (IC_{50}) for entacapone and tolcapone have been measured in Morgan 2013
  - Assumed noncompetitive BSEP and MRP inhibition
  - Used reported BSEP IC_{50} data as basis for noncompetitive BSEP Ki
  - Used reported MRP4 IC_{50} as basis for noncompetitive basolateral Ki

Compound	*in vitro* IC50 Data (Morgan 2013)	DILIsym® Parameter Name	DILIsym® Parameter Input
Entacapone	Human BSEP IC_{50} 55.6 uM	Ki_noncomp_ BSEP_CompY	1.7E-02 mg/mL
Entacapone	Human MRP4 IC_{50} 6.8 uM	Ki_noncomp_ baso_CompY	2.1E-03 mg/mL
Tolcapone	Human BSEP IC_{50} 36.6 uM	Ki_noncomp_ BSEP_CompY	1.0E-02 mg/mL
Tolcapone	Human MRP4 IC_{50} 16.7 uM	Ki_noncomp_ baso_CompY	4.6E-03 mg/mL

Entacapone MW: 305 Tolcapone MW: 273

Workflow for Modeling Entacapone and Tolcapone with MITOsym® and DILIsym®

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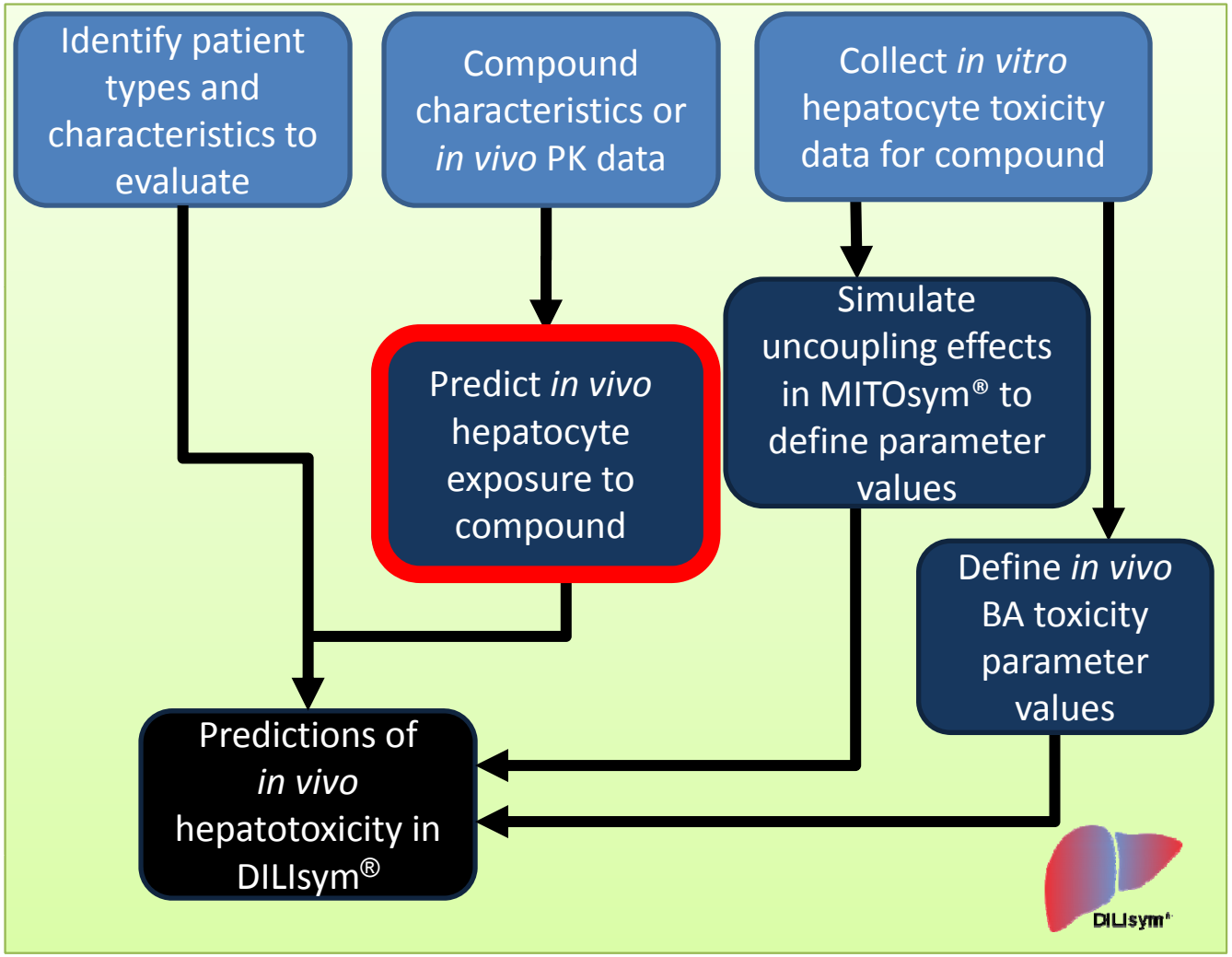
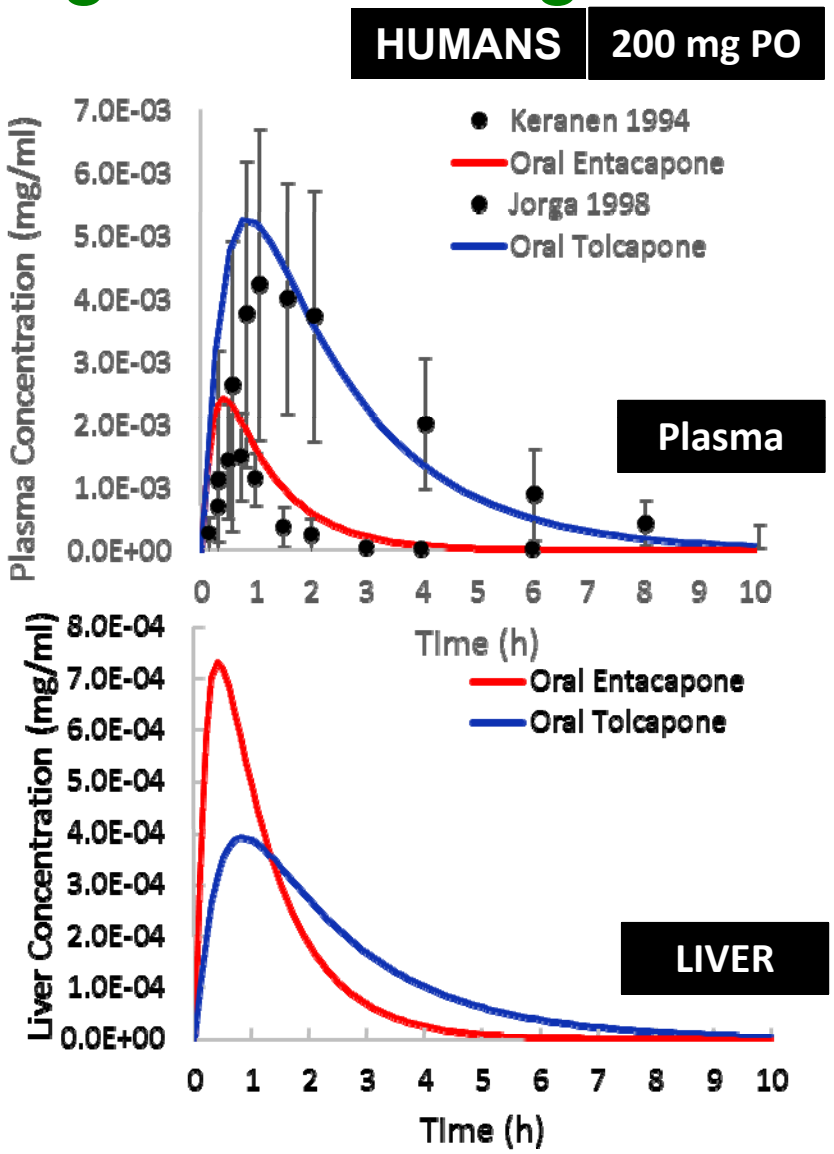


Entacapone vs. Tolcapone: Plasma and Liver Concentrations following Oral Dosing

- Similar ADME properties for Entacapone and Tolcapone
  - Low percentage of parent compound found in urine and feces samples
  - Both compounds are mainly metabolized in liver
  - Parent compounds responsible for liver toxicity, no reactive metabolites
- Used Compound Y structure in DILIsym®
  - Simpler than Compounds W and X
  - Multi-route and multi-dose time-series data
- Differences in PK between entacapone and tolcapone recapitulated by model
  - Lower Cmax and Tmax with entacapone
- Maintained 5-15% liver to plasma tolcapone ratio
  - Based on rat whole-body autoradiography results (Tasmar FDA filing documents)
- Maintained 30% liver to plasma entacapone ratio
  - Higher liver-to-plasma concentration ratio for entacapone in rat (Haasio et al 2002)



Clinical Data and Simulation Results

Workflow for Modeling Entacapone and Tolcapone with MITOsym® and DILIsym®

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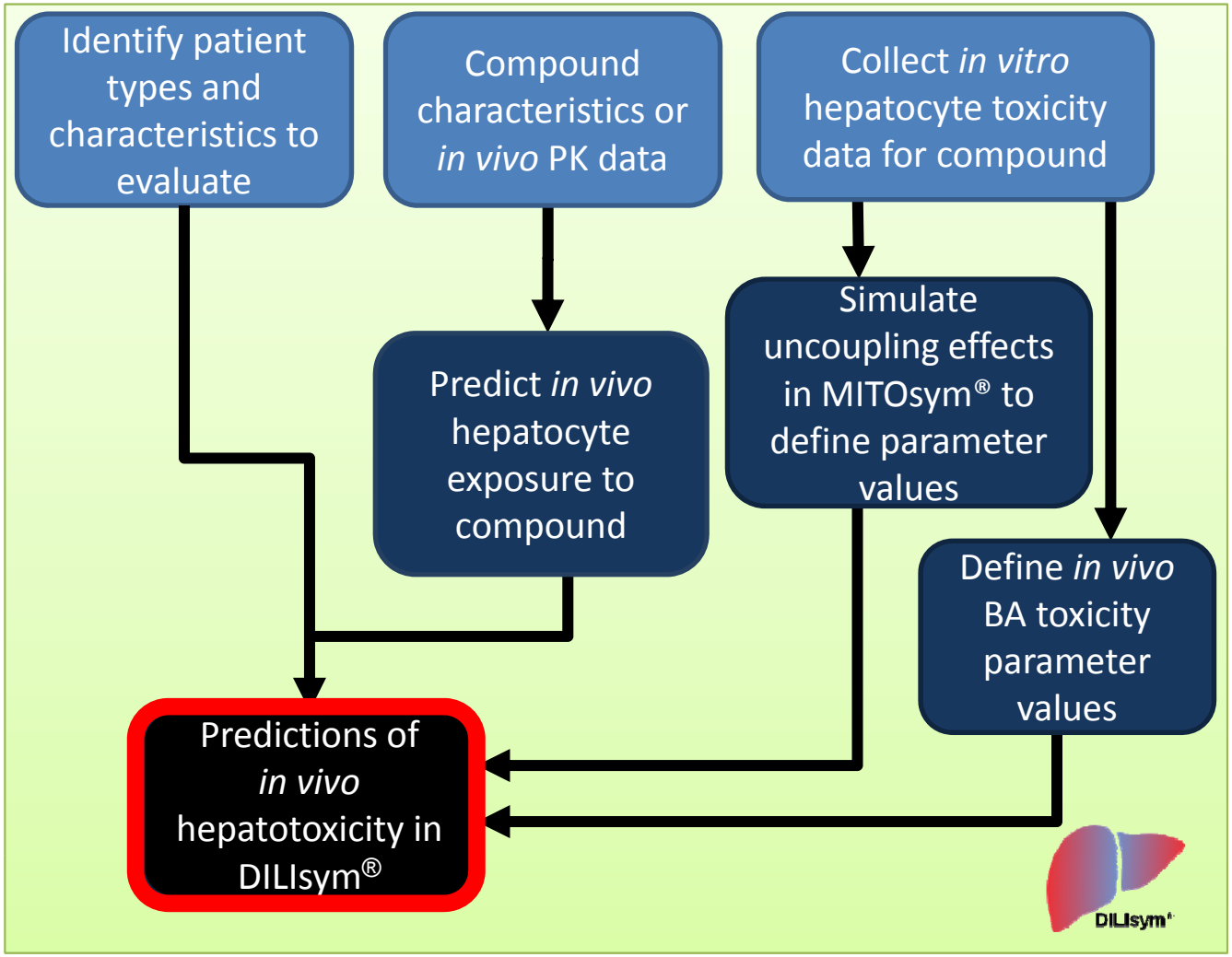
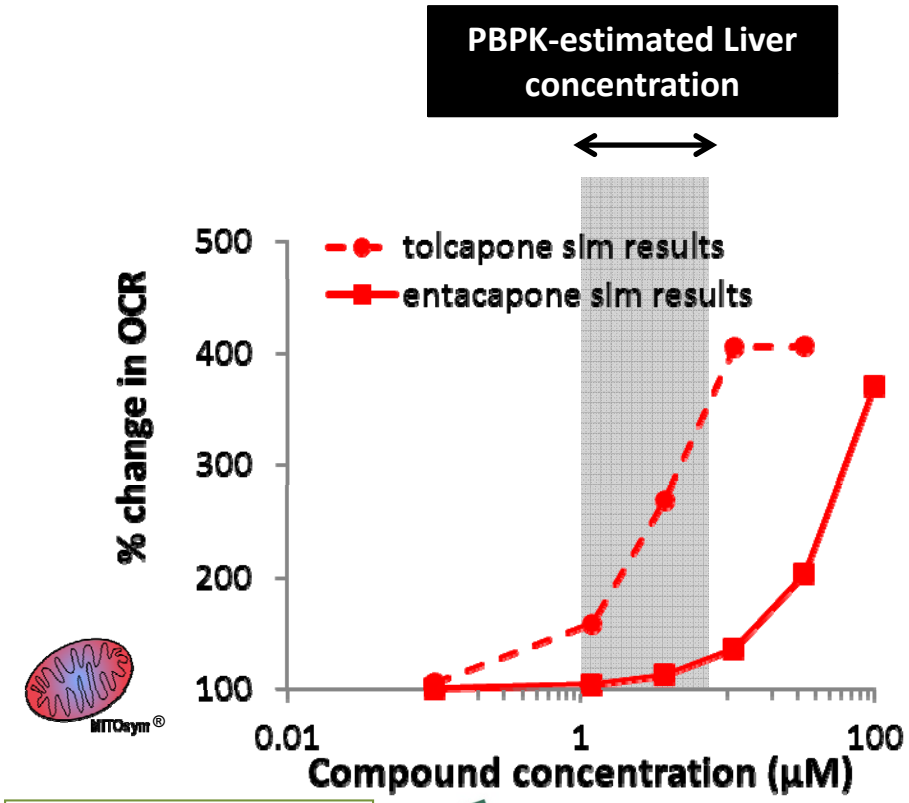
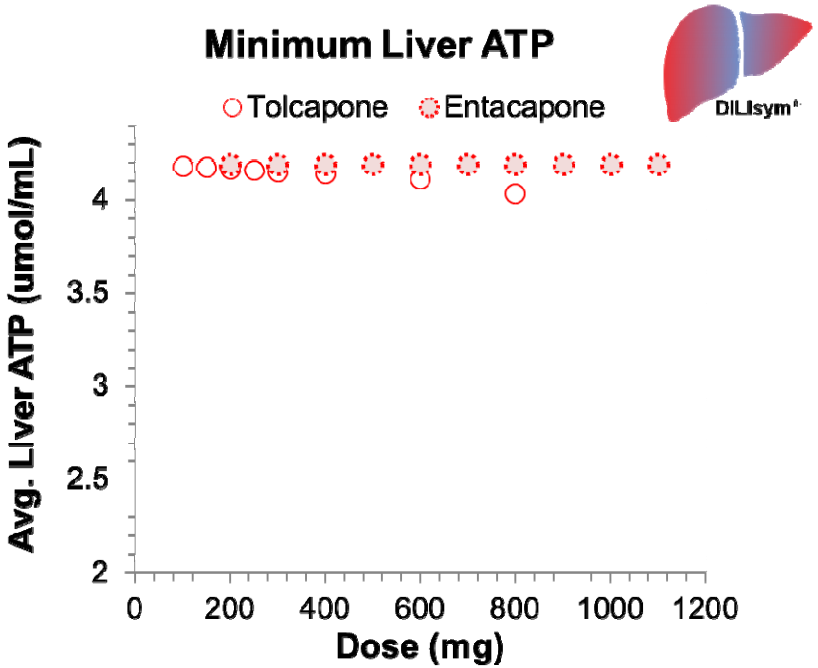
Baseline human and SimPops™



Simulated ATP Levels in Response to Entacapone and Tolcapone in the Baseline Human

- ATP-dose response simulation for entacapone and tolcapone
  - Entacapone Dose range 200~1200 mg, 8 times per day for one week
  - Tolcapone Dose range 100~800 mg, 8 times per day for one week
- Minor ATP reduction observed in tolcapone but no impact for entacapone

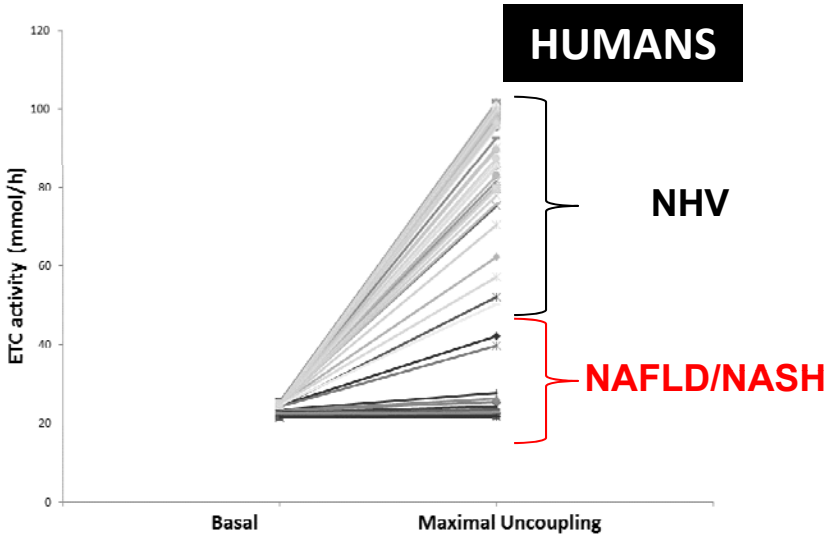


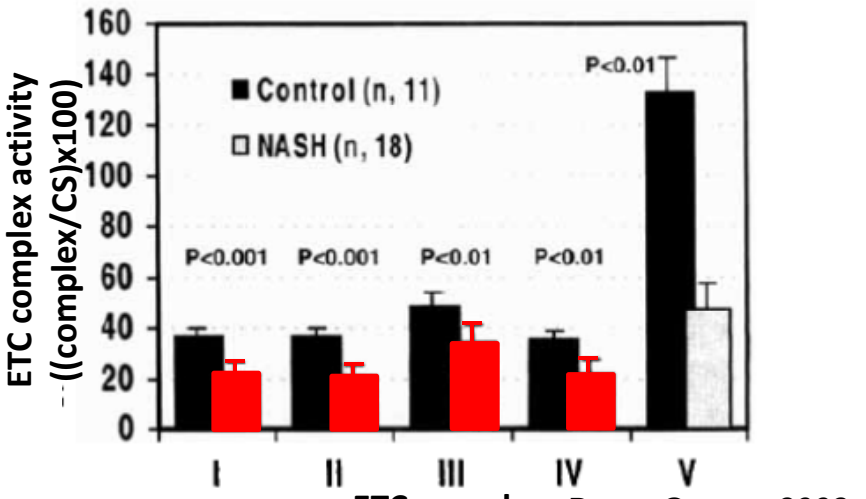


Simulation Results

BA-MITO SimPops™ Includes Patients with Compromised Mitochondria Characteristics

- SimPops™ generated with variability in mitochondria-related and other parameters
  - Human_mito_BA_v3A_6 (N=229)
  - Consistent with data from measured ETC complex activity from liver biopsies
  - Simulation results demonstrating respiratory reserve of each patient are displayed
- Generated simulated patients with normal to substantially compromised mitochondria function
  - Simulated patients basal ETC activity consistent with normal, healthy volunteers and NASH patients[1]
  - SimPops™ range consistent with mean +/- 2 SD from measured data[1]
  - Respiratory reserve scaling parameter also based on range of data from Perez-Carrera
  - In the absence of Western Blot data, the assumption is that the respiratory reserve scales similarly with basal ETC activity
- NASH incidence estimated to be 3-5%[2]
  - NAFLD incidence estimated to be 20%[3]
  - Declining liver mito function observed over time in high fat fed mice[4]
  - SimPops™ consistent with these distributions
  - N=17 (17/229 of Human_mito_BA_v3A_6)





Perez-Carrera 2003

[1]Perez-Carrera 2003, [2]Ruhl 2004, [3]Papandreou 2007, [4]Mantena 2009

in vivo Hepatotoxicity Profiles Assessed Using Human BA-MITO SimPops™

- No ALT elevations observed in simulations at the population level following oral administration with entacapone
  - Clinical protocol (up to 8 oral doses of 200mg)
  - None of the human SimPops™ exhibited serum ALT elevations greater than 3x ULN
  - Consistent with lack of clinical hepatotoxicity reported for entacapone
- Small percentage of simulated patients treated with tolcapone with elevated ALT
  - Consistent with infrequent clinical hepatotoxicity reported for tolcapone
  - 3% of patients in clinical trials had >3x ULN ALT
  - NAFLD/NASH simulated patients most responsive to tolcapone hepatotoxic effects
- Simulation results revealed BSEP transporter inhibition contributed minimal liver toxicity

HUMANS

Simulated with Human_mito_BA_v3A_6 SimPops™, n=229	Simulated ALT >3x ULN	Clinical Data
Entacapone 200mg oral 8xday 1 week	0/229 (0%)	0/1000s (0%)
Tolcapone 200mg oral TID 1 week	5/229 (2%)	8/293 (3%)

Summary

- Entacapone and tolcapone represent a “clean/toxic” compound pair with similar pharmacologic effects but differences in observed clinical hepatotoxicity
- *in vitro* data was used to define *in vivo* toxicity parameter values for both compounds
  - Uncoupling effects were simulated in MITOsym® and mitochondrial toxicity parameter values were translated to DILIsym® parameter values
  - Reported *in vitro* bile acid transport inhibition data was gathered and used to define DILIsym® toxicity parameters
  - Similar approaches can be applied to translate mitochondrial dysfunction data collected in primary cells
- Hepatotoxicity profiles for both compounds were simulated in the baseline human and within the human SimPops™
  - DILIsym® recaptured the differences in hepatotoxicity observed clinically based on mechanistic information
    - No injury was observed in simulations following treatment with entacapone
    - A small percentage of simulated patients treated with tolcapone had elevated ALT

DILIsym® Training Agenda – September 11, 2014

- 8:30 AM – Introduction
  - Training session goals
  - DILIsym® v3B overview and highlights
  - Model architecture notes
- 8:45 AM – Modeling troglitazone with DILIsym®
- 9:45 AM – Break
- 10:00 AM – Modeling entacapone and tolcapone with MITOsym® and DILIsym®
- 11:00 AM – Modeling compounds that disturb reactive oxygen species balance
- 11:30 AM – Lunch
- 12:30 PM – Discussion Topics
  - Data needs and use for PBPK modeling within DILIsym®
  - Free vs. total drug concentrations as determinants of toxicity mechanisms
  - DILIsym® equation design
  - *Biomarker design within DILIsym® (if time permits)*
  - *Timing of injury and injury progression within DILIsym® (if time permits)*
- 2:45 PM – Open discussion and wrap up
- 3:00 PM – Training concludes
  - DILI-sim modeling team is available for questions

Modeling Compounds that Disturb the Reactive Oxygen Species Balance Involves Two Primary Steps

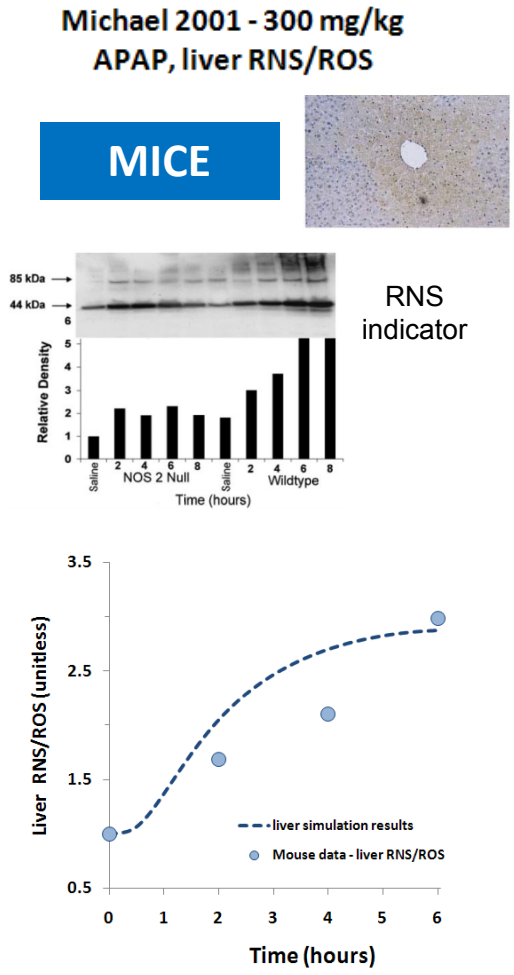
1. Step one: data gathering
   - *In vivo* assessments of reactive oxygen or nitrogen species (ROS/RNS)
   - *In vitro* assessments of reactive oxygen or nitrogen species (ROS/RNS)
     - Endpoints recommended by the DILIsym® team
     - Exposure range recommended by the DILIsym® team
     - Intracellular concentration assessments or estimates recommended by the DILIsym® team

2. Step two: translate data to DILIsym® parameters
   - Establish a dose response to use for the optimization
   - Implement an '*in vitro*' like environment within DILIsym® using Compound Y and optimize the simulations to match the *in vitro* data

- Characteristics of the ROS/RNS toxicity pathway within DILIsym®

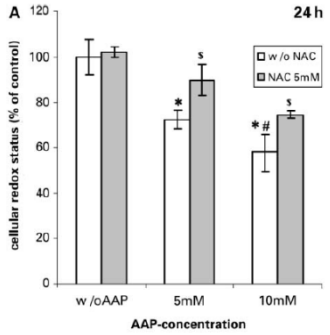
In vivo Assessments of ROS/RNS are Useful but Rarely Available

- *In vivo* assessments of ROS/RNS in the liver after drug exposure are sometimes (but rarely) available
- These assessments are useful for obtaining the necessary parameter within DILIsym® to represent ROS/RNS increases if:
  - A PBPK model has been established for the drug and species from which the data originate
  - The molecular entity (parent or metabolite) suspected of causing the ROS/RNS is represented in the liver within the PBPK model
    - e.g. NAPQI in an APAP PBPK model
- Simulations of ROS/RNS are fitted to the data
- This session will focus on the use of *in vitro* data
  - *In vivo* endpoints related to ROS/RNS are practically never available for compounds in development with multiple doses, time courses, etc.
  - *In vivo* endpoints related to ROS/RNS can be used for validation and refinement of the *in vitro* approach post-hoc if they become available during the latter stages of development



In vitro Indicators of ROS/RNS Recommended by the DILIsym® team

- Several *in vitro* indicators of ROS/RNS are available (examples listed here)
  - **Thiobarbituric acid reactive substances (TBARS), use malondialdehyde (MDA)**
  - **Peroxynitrite**
  - **Lipid hydroperoxide**
  - **Fluorescent probes (DCFDA, DHR123)**
  - **Mitochondrial superoxide (MitoSOX™ Red)**
- Each potential cell type carries positives and negatives (sample shown in table)
  - Ideally, one molecule can be directly connected to one effect in the experiment (mixtures complicated the parameterization)
  - Active transport is also a consideration
- The more time points measured, the more information garnered on the relationship between exposure and ROS/RNS
  - This is generally limited by budget
  - We recommend the following time points in order of decreasing importance
    - **24 hours**
    - **12 hours**
    - **6 hours**
    - **1 hour**



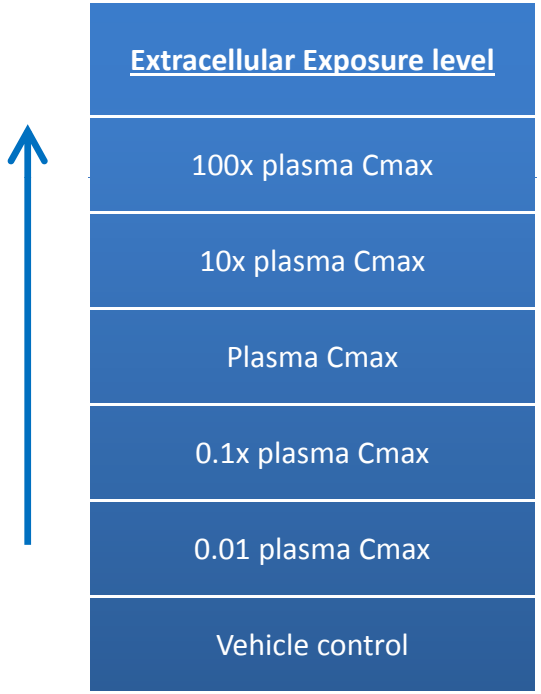
HepG2

Manov 2004

Cell Type	Pros	Cons
HepG2	Availability and cost; lack of drug metabolism if pure metabolites are available for testing individually	Lack of drug metabolism if pure metabolites are not available for testing individually
Primary hepatocytes	Drug metabolism if pure metabolites are not available for testing individually	Availability and cost; drug metabolism if pure metabolites are available for testing individually
HepaRG	Availability and cost; drug metabolism if pure metabolites are not available for testing individually	Drug metabolism if pure metabolites are available for testing individually

The Recommended Extracellular Exposure Range is Based on Relevant Plasma Concentrations *in vivo*

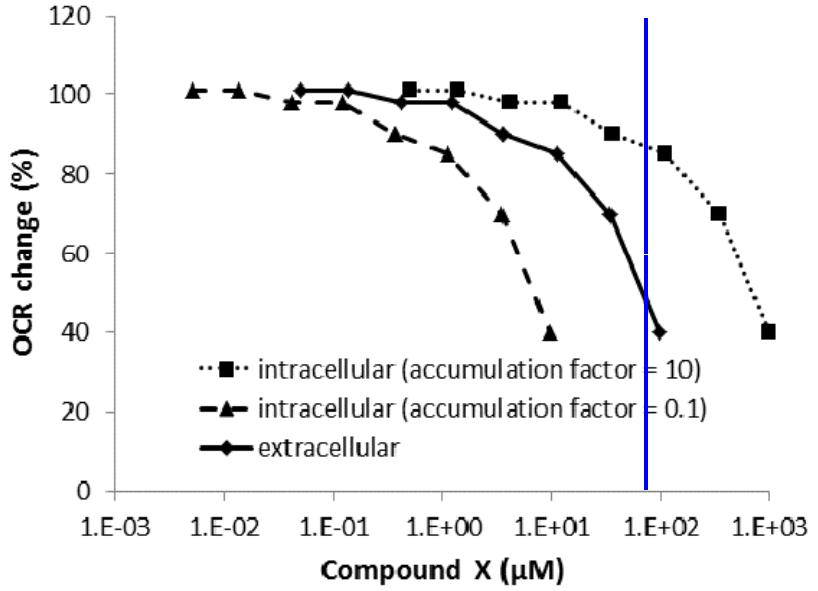
- Extracellular exposure ranges should be relative to the plasma concentrations, or predictions thereof
  - **This is critical to gathering useful data!**
  - *In vitro* studies are often done at extremely high concentrations
  - Contrary to the approach often taken, pushing the cells to a maximal response is not the best approach for this application
- Example table shown that blankets the predicted C_{max}
- Range should be adjusted based on predicted variability
  - High anticipated variability in exposure warrants a broader range
  - May have to make judgment call based on early *in vitro* indicators



Improved DILIsym® Parameter Values When Intracellular Compound Is Measured

- Most Seahorse oxygen consumption rate (OCR) or ROS/RNS data are expressed in an exposure-response relationship
  - OCR change on y-axis
  - Extracellular compound concentration on x-axis
- Numerous compounds have been shown to accumulate in liver
  - Potency relative to intracellular concentrations different than relative to extracellular
  - Intracellular ≠ extracellular
- Basing parameter values on extracellular concentrations introduces inaccuracy for compounds that accumulate in hepatocytes
- Recommend measuring intracellular compound concentration for cell based assays used to provide DILIsym® parameter values
  - OCR, ROS production
  - For compounds that are known to have liver:blood ratio ≠ 1 (or not known)

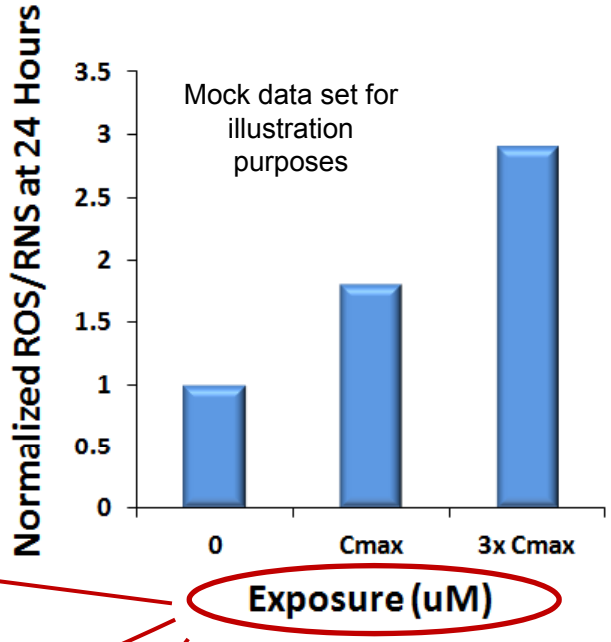
HepG2



	extracellular	intracellular
10X accumulation	74.4 uM	744 uM
1x accumulation	74.4 uM	74.4 uM
0.1x accumulation	74.4 uM	7.29 uM

Establishing a Dose Response Requires Choosing an Exposure Estimate and Preferred Time Point(s)

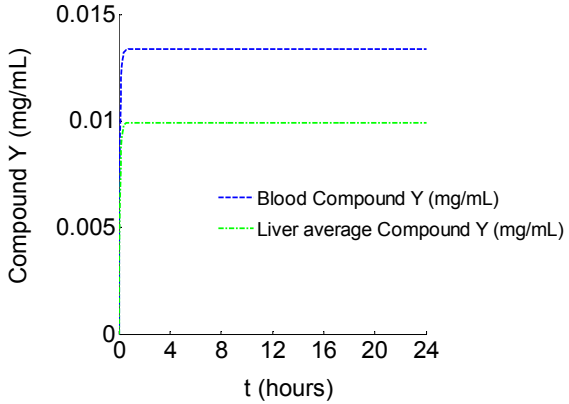
- A relationship between exposure and normalized ROS/RNS response at a single time point is typically used
  - Could also choose to fit multiple time courses
- Intracellular exposure can be estimated in three ways
  - Measured (see previous slide); this is the recommended method, but is not always possible
  - Estimated using media concentration and PBPK parameters such as liver to blood concentration ratio
  - Assumed from media concentration; this is the least optimal approach, in our opinion



Compound Y within DILIsym® is Used to Setup an "*in vitro*–like" Simulation

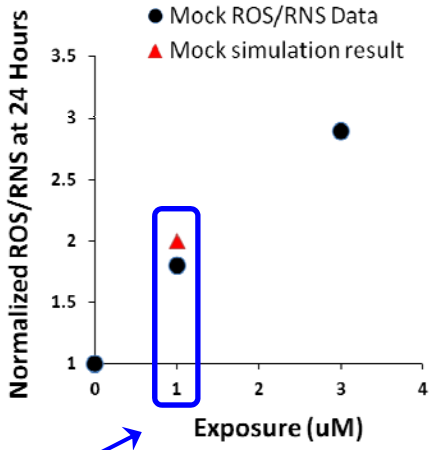
- Key attributes of the simulation setup
  - Start with Blank parameter set
  - IV infusion with Compound Y
  - Rapid uptake and dissolution of the drug
  - Rapid steady state in the blood and liver
  - Parameters altered shown in table
  - Compound Y parameter set will also be downloadable from www.DILIsym.com
    - "**Parameters_ROS_parameterization_CompY_SteadyState**"
- Constant liver exposure mimics the *in vitro* environment
- Example blood and liver concentrations shown

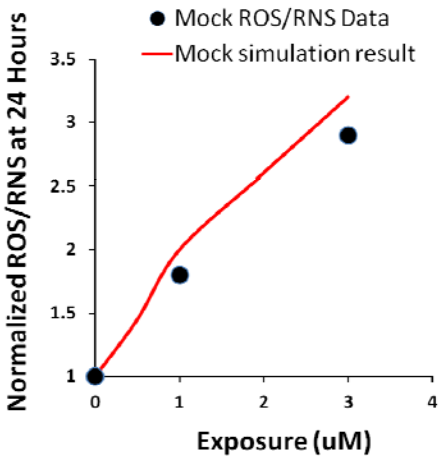
DILIsym® Parameter	Value
Species_check	Change to species of interest (blank default is human)
CompY_mech	Turn ROS/RNS production on for Compound Y
CompY_hepatic_cl	1000
kab_Comp_Y_IV	1000
Comp_Y_Vd	10
Comp_Y_mg_mol	Inverse of MW of compound
Comp_Y_mol_mg	MW of compound



Reproduce the Data Gathered with the "*in vitro*–like" Simulation Setup

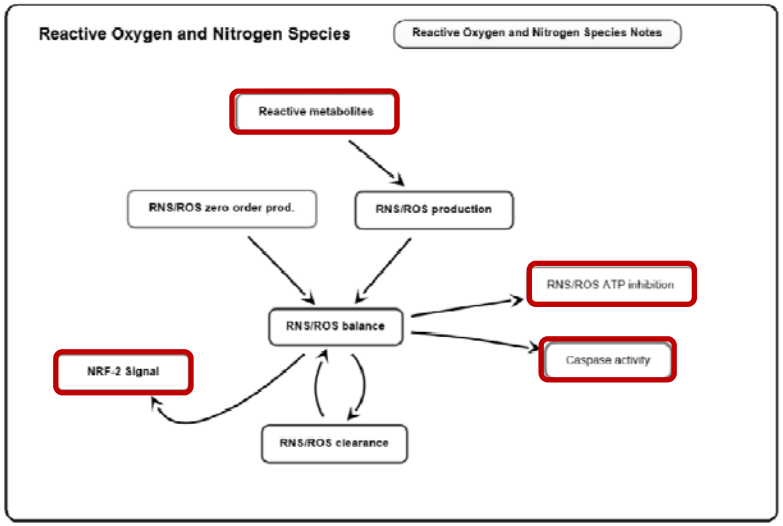
- The optimization process only involves one parameter, which is at the top of the in the 'Drug toxicity parameters' sub-set
  - **RNS_ROS_prod_const**
- Tune the Compound Y infusion rate to get exposures in the liver in the range of the *in vitro* exposure estimates
- Pick an exposure level and use the parameter sweep tool within DILIsym® to find a RNS_ROS_prod_const value that gives a reasonable level of ROS at the corresponding time point; output to use for ROS is 'Liver average RNS/ROS'
- Use the parameter sweep tool with the value of RNS_ROS_prod_const found above to run an exposure sweep across the exposure range measured
  - Iterate as necessary
- **Overall, find the value of RNS_ROS_prod_const that matches the data**





Characteristics of the ROS/RNS Toxicity Pathway within DILIsym®

- Any drug or metabolite within DILIsym® can be selected to cause increased RNS/ROS production
- ROS currently has two toxicity pathways within DILIsym® :
  - Inhibits ATP production, leading to necrosis when ATP is significantly depleted
  - Increases caspase activation, which leads to apoptosis (as of v3B)
- Caspase activation occurs at lower ROS levels than ROS levels that cause necrosis
- ROS also activates the NRF-2 pathway
- ROS is transported across zones of the liver and causes injury propagation (based on a first-order, gradient driven equation)
  - See JDesigner Notes for details
- ROS production increases within DILIsym® are represented with a linear, first-order equation
  - AUC is more critical than C_{max}



DILIsym® Training Agenda – September 11, 2014

- 8:30 AM – Introduction
  - Training session goals
  - DILIsym® v3B overview and highlights
  - Model architecture notes
- 8:45 AM – Modeling troglitazone with DILIsym®
- 9:45 AM – Break
- 10:00 AM – Modeling entacapone and tolcapone with MITOsym® and DILIsym®
- 11:00 AM – Modeling compounds that disturb reactive oxygen species balance
- 11:30 AM – Lunch
- 12:30 PM – Discussion Topics
  - Data needs and use for PBPK modeling within DILIsym®
  - Free vs. total drug concentrations as determinants of toxicity mechanisms
  - DILIsym® equation design
  - *Biomarker design within DILIsym® (if time permits)*
  - *Timing of injury and injury progression within DILIsym® (if time permits)*
- 2:45 PM – Open discussion and wrap up
- 3:00 PM – Training concludes
  - DILI-sim modeling team is available for questions

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More *In Vivo* Data Means Less Emphasis on *In Vitro* Data in Model Parameter Selection

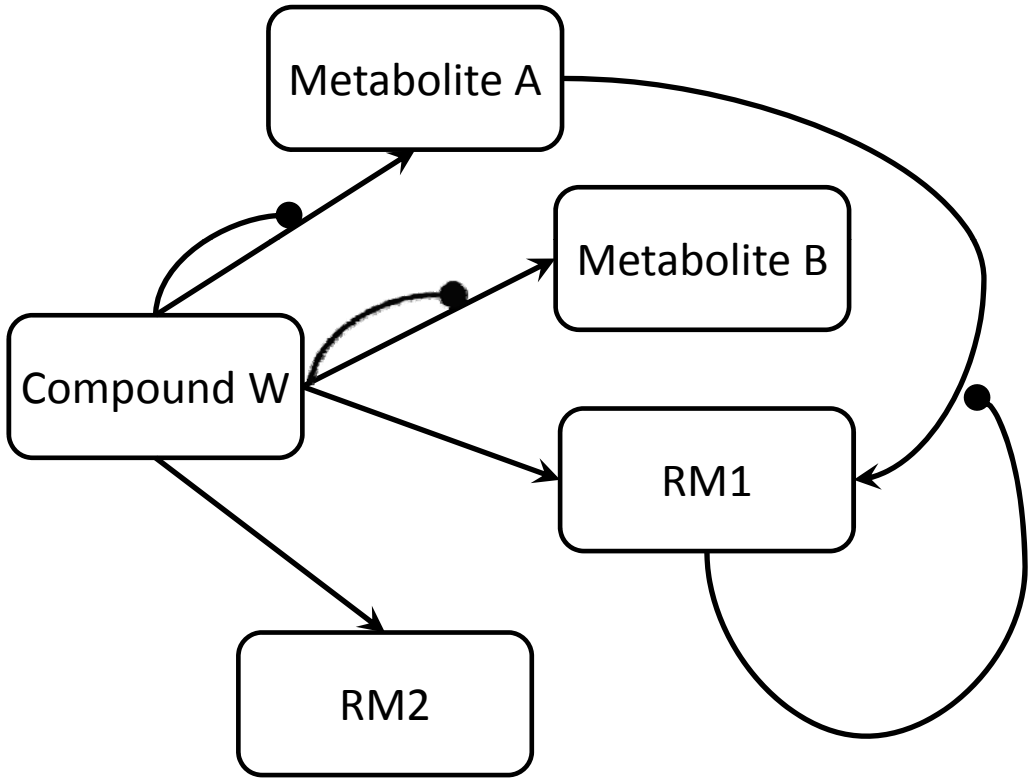
- More PK data sets available for use during model parameterization means less emphasis on *in vitro* data
  - Difficult to optimize a large number of parameters to a single data set
- Different kinds of data sets are important for model parameterization
  - Metabolite data are necessary if optimization of metabolism parameters is needed
  - Single and multiple dose data necessary to accurately represent clearance dynamics
  - Liver/blood concentration ratios necessary to represent partition coefficient
- *In vitro* data are still useful for full confidence in PBPK model
  - Presence of active transport into liver generally determined through *in vitro* assay
  - Induction/suppression of uptake and metabolism also determined *in vitro*
  - Other *in vitro* data can be used to constrain optimization process
  - Most important aspect is to fit all available *in vivo* PBPK data

Selecting Proper PBPK Parameters Is Necessary to Get Maximum Value from DILIsym® Simulations

- Liver concentration dynamics are important for the accurate prediction of toxicity
- DILIsym® contains 132 PBPK parameters for the Compound W and X models
- PBPK model parameterization requires two main steps
  - Selecting appropriate metabolic scaffold
  - Parameterizing model

Parent Compound	Metabolite A	Reactive Metabolite 1
Comp_W_bil_cl	CompW_Met_A_bil_cl	GSH_CompW_RM1_fu_L
Comp_W_fr_recir	CompW_Met_A_fr_recir	GSH_CompW_RM1_fu_P
Comp_W_B_P	CompW_Met_A_fu_L	GSH_CompW_RM1_L_B
Comp_W_G_B	CompW_Met_A_fu_P	GSH_CompW_RM1_Vd_wt
Comp_W_L_B	CompW_Met_A_B_P	k_CompW_RM1_GSH
Comp_W_Vmax_L_B	CompW_Met_A_L_B	k_CompW_RM1_protein
Comp_W_Km_L_B	CompW_Met_A_mg_mol	k_CompW_RM1_deactivation
tau_CompW_uptake_delay	CompW_Met_A_mol_mg	Vmax_CompW_RM1
CompW_uptake_induction_Vmax	CompW_Met_A_renal_cl	Km_CompW_RM1
CompW_uptake_induction_Km	CompW_Met_A_Vd_wt	CompW_RM1_inhib_start_time
CompW_uptake_induction_Hill	Km_CompW_Met_A	CompW_RM1_inhib_stop_time
Comp_W_perm	Vmax_CompW_Met_A	CompW_RM1_inhib_percent
Comp_W_M_B	tau_CompW_MetA_delay	Km_CompW_RM1_adduct_transport
Comp_W_O_B	CompW_MetA_induction_Vmax	Vmax_CompW_RM1_adduct_transport
Comp_W_fu_G	CompW_MetA_induction_Km	CompW_RM1_mg_mol
Comp_W_fu_L	CompW_MetA_induction_Hill	CompW_RM1_mol_mg
Comp_W_fu_M	CL_PP_act_CompW_Met_A	CompW_RM1_adduct_half
Comp_W_fu_O	ML_PP_act_CompW_Met_A	PP_PP_act_CompW_RM1
Comp_W_fu_P	PP_PP_act_CompW_Met_A	CL_PP_act_CompW_RM1
Fu_correlation_Comp_W		ML_PP_act_CompW_RM1
Comp_W_fu_corr_2		k_CompW_RM1_adduct_macro
Comp_W_fu_corr_1		k_CompW_RM1_adduct_liver_blood
Comp_W_fu_corr_0		Vmax_CompW_RM1_from_MetA
Comp_W_mg_mol		Km_CompW_RM1_from_MetA
Comp_W_mol_mg		CompW_MetA_RM1_inhib_Vmax
Comp_W_renal_cl		CompW_MetA_RM1_inhib_Ki
kdiss_Comp_W		k_enzyme_turnover_CompW
kge_Comp_W		
kab_Comp_W_oral		
Vmax_Comp_W_ab		
Km_Comp_W_ab		
k_out_gut_Comp_W		
kab_conj_Comp_W		
kab_Comp_W_IP		
kIV_Comp_W		

DILIsym® Can Represent Up To Four Metabolites In Addition to Parent Compound



- Selecting the proper metabolism scaffold requires:
  - Knowing what data are available for each metabolite
  - Knowing what chemical species are likely to be involved in toxic mechanisms
- Example: bosentan
  - Two main metabolites, neither reactive
  - Minor metabolite involved in toxicity
  - Parent compound induces metabolism

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Comp_W_B_P	CompW_Met_A_fu_L	GSH_CompW_RM1_L_B
Comp_W_G_B	CompW_Met_A_fu_P	GSH_CompW_RM1_Vd_wt
Comp_W_L_B	CompW_Met_A_B_P	k_CompW_RM1_GSH
Comp_W_Vmax_L_B	CompW_Met_A_L_B	k_CompW_RM1_protein
Comp_W_Km_L_B	CompW_Met_A_mg_mol	k_CompW_RM1_deactivation
tau_CompW_uptake_delay	CompW_Met_A_mol_mg	Vmax_CompW_RM1
CompW_uptake_induction_Vmax	CompW_Met_A_renal_cl	Km_CompW_RM1
CompW_uptake_induction_Km	CompW_Met_A_Vd_wt	CompW_RM1_inhib_start_time
CompW_uptake_induction_Hill	Km_CompW_Met_A	CompW_RM1_inhib_stop_time
Comp_W_perm	Vmax_CompW_Met_A	CompW_RM1_inhib_percent
Comp_W_M_B	tau_CompW_MetA_delay	Km_CompW_RM1_adduct_transport
Comp_W_O_B	CompW_MetA_induction_Vmax	Vmax_CompW_RM1_adduct_transport
Comp_W_fu_G	CompW_MetA_induction_Km	CompW_RM1_mg_mol
Comp_W_fu_L	CompW_MetA_induction_Hill	CompW_RM1_mol_mg
Comp_W_fu_M	CL_PP_act_CompW_Met_A	CompW_RM1_adduct_half
Comp_W_fu_O	ML_PP_act_CompW_Met_A	PP_PP_act_CompW_RM1
Comp_W_fu_P	PP_PP_act_CompW_Met_A	CL_PP_act_CompW_RM1
Fu_correlation_Comp_W		ML_PP_act_CompW_RM1
Comp_W_fu_corr_2		k_CompW_RM1_adduct_macro
Comp_W_fu_corr_1		k_CompW_RM1_adduct_liver_blood
Comp_W_fu_corr_0		Vmax_CompW_RM1_from_MetA
Comp_W_mg_mol		Km_CompW_RM1_from_MetA
Comp_W_mol_mg		CompW_MetA_RM1_inhib_Vmax
Comp_W_renal_cl		CompW_MetA_RM1_inhib_Ki
kdiss_Comp_W		k_enzyme_turnover_CompW
kge_Comp_W		
kab_Comp_W_oral		
Vmax_Comp_W_ab		
Km_Comp_W_ab		
k_out_gut_Comp_W		
kab_conj_Comp_W		
kab_Comp_W_IP		
kIV_Comp_W		

DILIsym® PBPK Input Parameters Fall into Several Main Categories

- Absorption
  - Active and passive models
- Distribution
  - Partition coefficients and fractions unbound
  - Active uptake model for liver
  - Partition and volume of distribution for metabolites
- Metabolism
  - Michaelis-Menten kinetics from parent to metabolites
  - RM reactions with GSH and protein
- Excretion
  - Biliary and renal clearance of parent and main metabolites
  - Clearance of protein adducts

Parent Compound	Metabolite A	Reactive Metabolite 1
Comp_W_bil_cl	CompW_Met_A_bil_cl	GSH_CompW_RM1_fu_L
Comp_W_fr_recir	CompW_Met_A_fr_recir	GSH_CompW_RM1_fu_P
Comp_W_B_P	CompW_Met_A_fu_L	GSH_CompW_RM1_L_B
Comp_W_G_B	CompW_Met_A_fu_P	GSH_CompW_RM1_Vd_wt
Comp_W_L_B	CompW_Met_A_B_P	k_CompW_RM1_GSH
Comp_W_Vmax_L_B	CompW_Met_A_L_B	k_CompW_RM1_protein
Comp_W_Km_L_B	CompW_Met_A_mg_mol	k_CompW_RM1_deactivation
tau_CompW_uptake_delay	CompW_Met_A_mol_mg	Vmax_CompW_RM1
CompW_uptake_induction_Vmax	CompW_Met_A_renal_cl	Km_CompW_RM1
CompW_uptake_induction_Km	CompW_Met_A_Vd_wt	CompW_RM1_inhib_start_time
CompW_uptake_induction_Hill	Km_CompW_Met_A	CompW_RM1_inhib_stop_time
Comp_W_perm	Vmax_CompW_Met_A	CompW_RM1_inhib_percent
Comp_W_M_B	tau_CompW_MetA_delay	Km_CompW_RM1_adduct_transport
Comp_W_O_B	CompW_MetA_induction_Vmax	Vmax_CompW_RM1_adduct_transport
Comp_W_fu_G	CompW_MetA_induction_Km	CompW_RM1_mg_mol
Comp_W_fu_L	CompW_MetA_induction_Hill	CompW_RM1_mol_mg
Comp_W_fu_M	CL_PP_act_CompW_Met_A	CompW_RM1_adduct_half
Comp_W_fu_O	ML_PP_act_CompW_Met_A	PP_PP_act_CompW_RM1
Comp_W_fu_P	PP_PP_act_CompW_Met_A	CL_PP_act_CompW_RM1
Fu_correlation_Comp_W		ML_PP_act_CompW_RM1
Comp_W_fu_corr_2		k_CompW_RM1_adduct_macro
Comp_W_fu_corr_1		k_CompW_RM1_adduct_liver_blood
Comp_W_fu_corr_0		Vmax_CompW_RM1_from_MetA
Comp_W_mg_mol		Km_CompW_RM1_from_MetA
Comp_W_mol_mg		CompW_MetA_RM1_inhib_Vmax
Comp_W_renal_cl		CompW_MetA_RM1_inhib_Ki
kdiss_Comp_W		k_enzyme_turnover_CompW
kge_Comp_W		
kab_Comp_W_oral		
Vmax_Comp_W_ab		
Km_Comp_W_ab		
k_out_gut_Comp_W		
kab_conj_Comp_W		
kab_Comp_W_IP		
kIV_Comp_W		

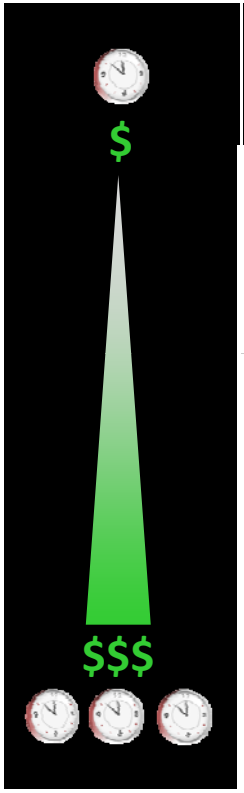
Method for Determining Distribution Parameters Depends on Data Available

- Distribution parameters can be determined from either *in vivo* or *in vitro* data
  - Physicochemical properties
  - *In vitro* cellular uptake assays
  - Animal WBAR studies

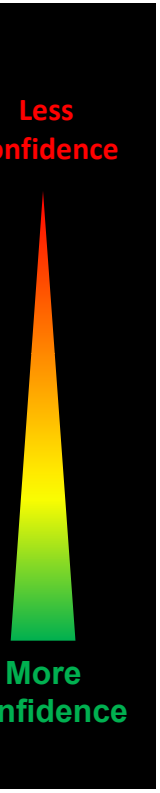
- Input panel document provides some insight into most useful assays for best DILIsym® inputs

Parent Compound	Metabolite A
Comp_W_bil_cl	CompW_Met_A_bil_cl
Comp_W_fr_recir	CompW_Met_A_fr_recir
Comp_W_B_P	CompW_Met_A_fu_L
Comp_W_G_B	CompW_Met_A_fu_P
Comp_W_L_B	CompW_Met_A_B_P
Comp_W_Vmax_L_B	CompW_Met_A_L_B
Comp_W_Km_L_B	CompW_Met_A_mg_mol
tau_CompW_uptake_delay	CompW_Met_A_mol_mg
CompW_uptake_induction_Vmax	CompW_Met_A_renal_cl
CompW_uptake_induction_Km	CompW_Met_A_Vd_wt
CompW_uptake_induction_Hill	Km_CompW_Met_A
Comp_W_perm	Vmax_CompW_Met_A
Comp_W_M_B	tau_CompW_MetA_delay
Comp_W_O_B	CompW_MetA_induction_Vmax
Comp_W_fu_G	CompW_MetA_induction_Km
Comp_W_fu_L	CompW_MetA_induction_Hill
Comp_W_fu_M	CL_PP_act_CompW_Met_A
Comp_W_fu_O	ML_PP_act_CompW_Met_A
Comp_W_fu_P	PP_PP_act_CompW_Met_A
Fu_correlation_Comp_W	
Comp_W_fu_corr_2	
Comp_W_fu_corr_1	
Comp_W_fu_corr_0	
Comp_W_mg_mol	
Comp_W_mol_mg	
Comp_W_renal_cl	
kdiss_Comp_W	
kge_Comp_W	
kab_Comp_W_oral	
Vmax_Comp_W_ab	
Km_Comp_W_ab	
k_out_gut_Comp_W	
kab_conj_Comp_W	
kab_Comp_W_IP	
kIV_Comp_W	

Basic Molecular Properties

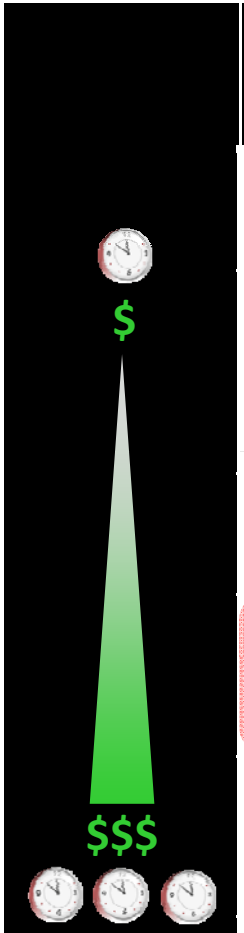


Inputs or Data Needed	Commonly Used Systems/Assays	DILIsym® Preferred Systems
Acid or base?	Standard	Industry standard
Monoprotoic or diprotic	Standard	Industry standard
pKa(s)	Standard	Industry standard
log P (oil:water and octanol:water)	Standard	Industry standard
Fraction bound to plasma or serum proteins	Standard	Industry standard
Fraction partitioned into red blood cells	Standard	Industry standard

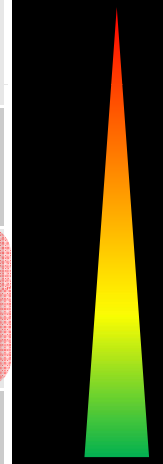


Drug Clearance and Transport

M - Metabolite analytics required



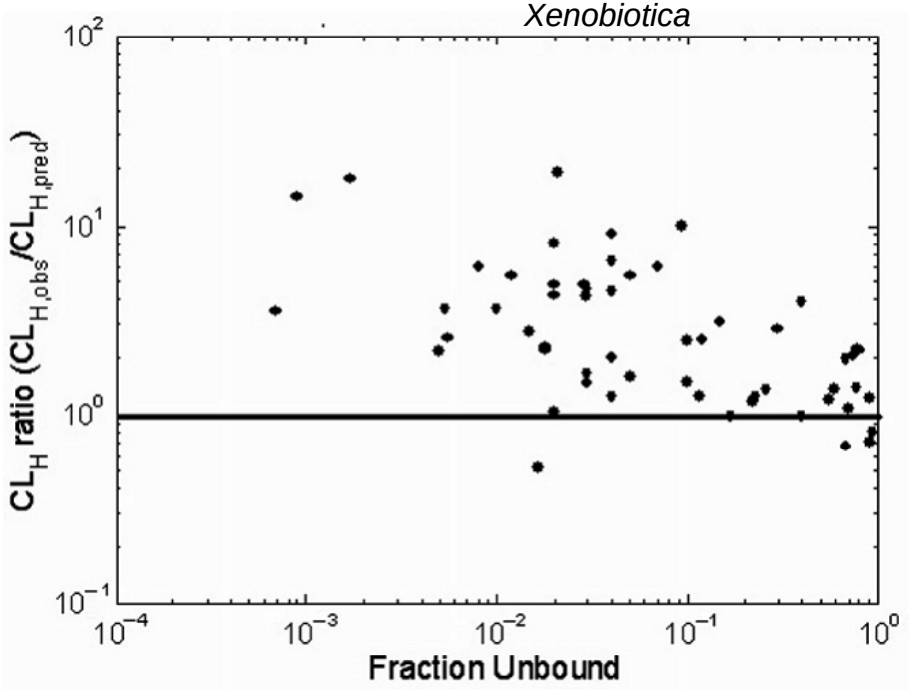
Inputs or Data Needed	Commonly Used Systems/Assays	DILIsym® Preferred Systems
Rate of metabolic clearance of parent compound	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes to distinguish ph I and ph II pathways
Metabolic clearance of parent compound by specific pathways	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes with co-factors to get quantitative estimate of glucuronidation, sulfation, CYP450 pathway ratios
Mechanism based cytochrome P450 inhibition (drug-drug interaction (DDI) activity)	IC50 value or Ki; reference FDA guidance on DDI's	Industry standard
Potential for active transport in the liver (rate of hepatocyte uptake); transport kinetic information if possible	Hepatocyte suspensions; sandwich cultured (SC) hepatocytes; vesicles	Hepatocyte suspensions
Metabolic clearance of parent compound and coincidental appearance of specific metabolites (M)	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes to distinguish ph I and ph II pathways
Metabolic clearance of parent compound and coincidental rate of appearance of specific metabolites (M)	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes with co-factors to get quantitative estimate of glucuronidation, sulfation, CYP450 pathway ratios
Parent distribution, clearance, and metabolite formation kinetics	*in vivo*	*in vivo*; radiolabel mass balance data preferred



Determination of Fraction Unbound Requires Both *In Vitro* Data and Optimization

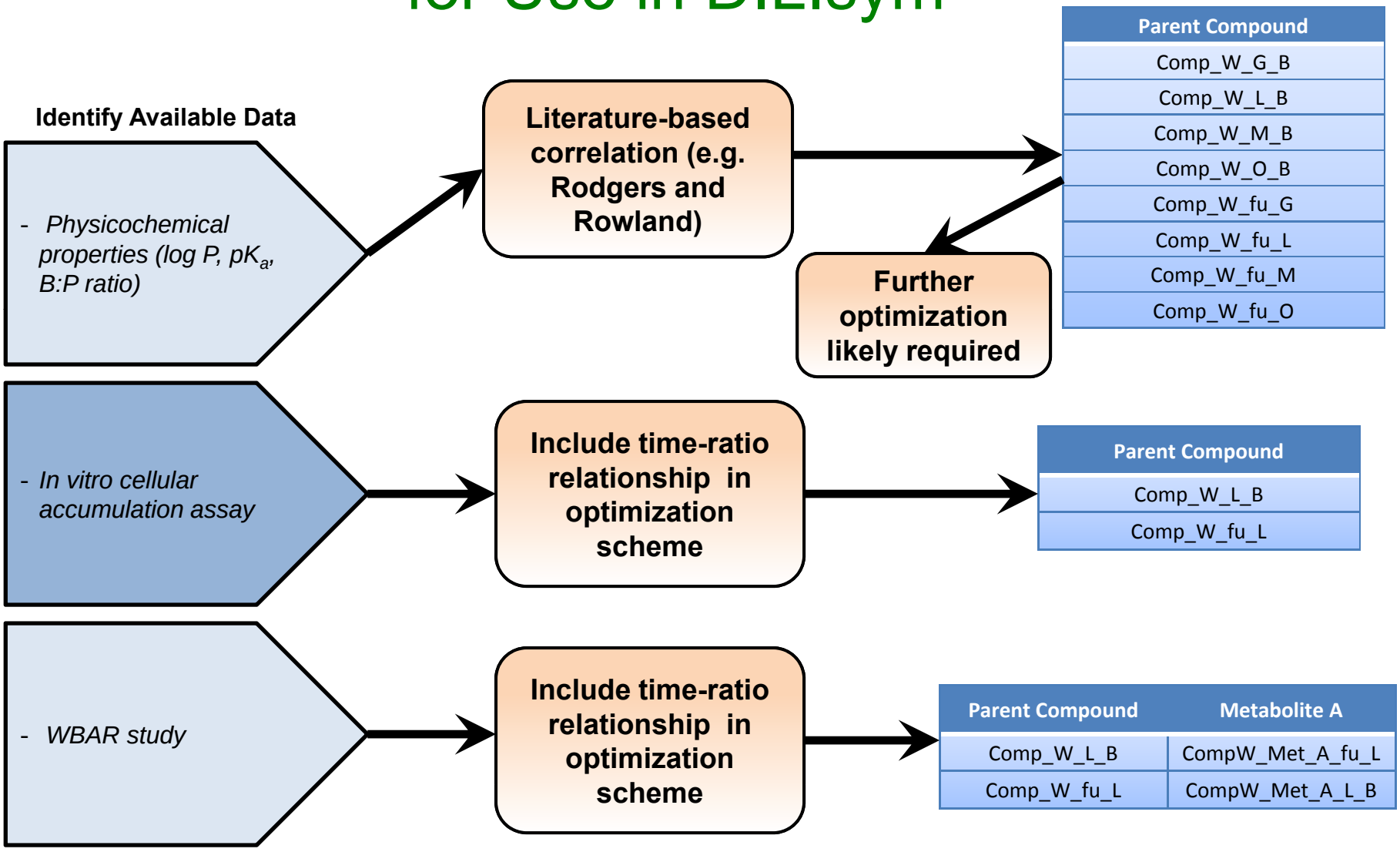
- Baker (2007) demonstrated that using plasma fraction unbound from *in vitro* assays can significantly underestimate the amount of drug available for distribution or clearance
  - Especially true when $f_{u,p} < 0.1$
  - Due to non-equilibrium conditions of protein binding *in vivo* where binding affinity must be considered
- *In vitro* value alone may not allow ideal prediction of PK data
  - *In vitro* value is a good starting point
  - Fit $f_{u,p}$ to data if dynamics cannot be matched with *in vitro* value
- Plasma fraction unbound can vary with plasma concentration
  - Option for this is included in DILIsym: fu_correlation
- Reminder: fraction unbound in plasma is used in calculation to determine $f_{u,t}$ from partition coefficients
  - $f_{u,t}$ is what DILIsym® uses to determine partitioning

Baker (2007)
Xenobiotica

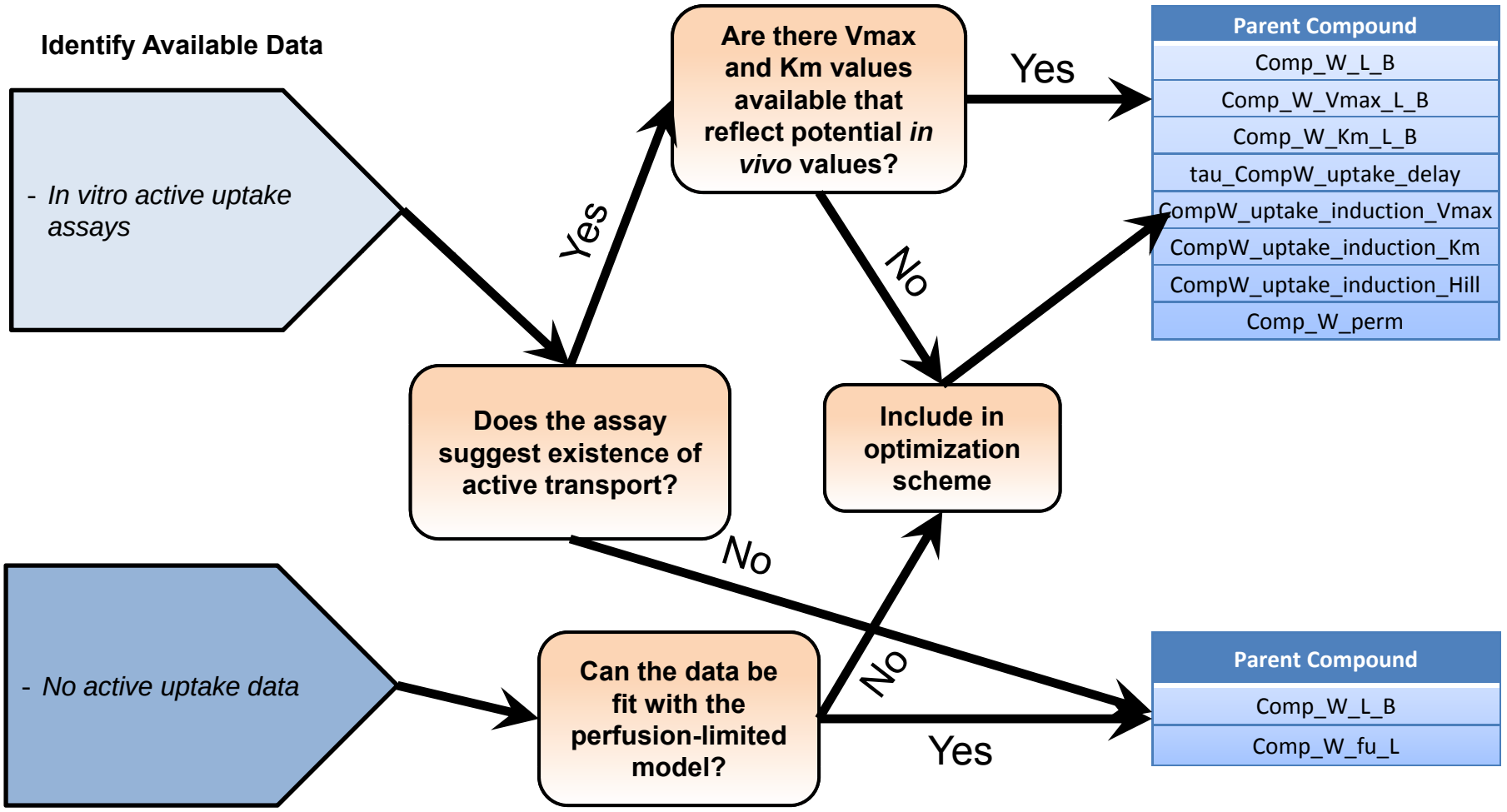


$$f_{u,t} = \frac{f_{u,p}}{K_t}$$

Predicting Partition Coefficients for Use in DILIsym®



Selecting the DILIsym® Parameters to Use for Active Liver Uptake



Method for Determining Metabolism Parameters Depends on Data Available

- Metabolism parameters can be determined from *in vitro* data or by fitting to PK data
  - *In vitro* microsome data
  - Hepatocyte metabolic clearance data
  - PK data **including complete metabolite time course in plasma**
- Input panel document provides some insight into most useful assays for best DILIsym® inputs

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tau_CompW_uptake_delay	CompW_Met_A_mol_mg
CompW_uptake_induction_Vmax	CompW_Met_A_renal_cl
CompW_uptake_induction_Km	CompW_Met_A_Vd_wt
CompW_uptake_induction_Hill	Km_CompW_Met_A
Comp_W_perm	Vmax_CompW_Met_A
Comp_W_M_B	tau_CompW_MetA_delay
Comp_W_O_B	CompW_MetA_induction_Vmax
Comp_W_fu_G	CompW_MetA_induction_Km
Comp_W_fu_L	CompW_MetA_induction_Hill
Comp_W_fu_M	CL_PP_act_CompW_Met_A
Comp_W_fu_O	ML_PP_act_CompW_Met_A
Comp_W_fu_P	PP_PP_act_CompW_Met_A
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Comp_W_mg_mol	
Comp_W_mol_mg	
Comp_W_renal_cl	
kdiss_Comp_W	
kge_Comp_W	
kab_Comp_W_oral	
Vmax_Comp_W_ab	
Km_Comp_W_ab	
k_out_gut_Comp_W	
kab_conj_Comp_W	
kab_Comp_W_IP	
kIV_Comp_W	

Drug Clearance and Transport

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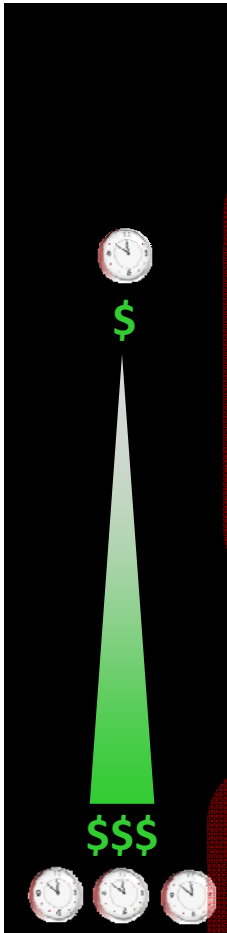

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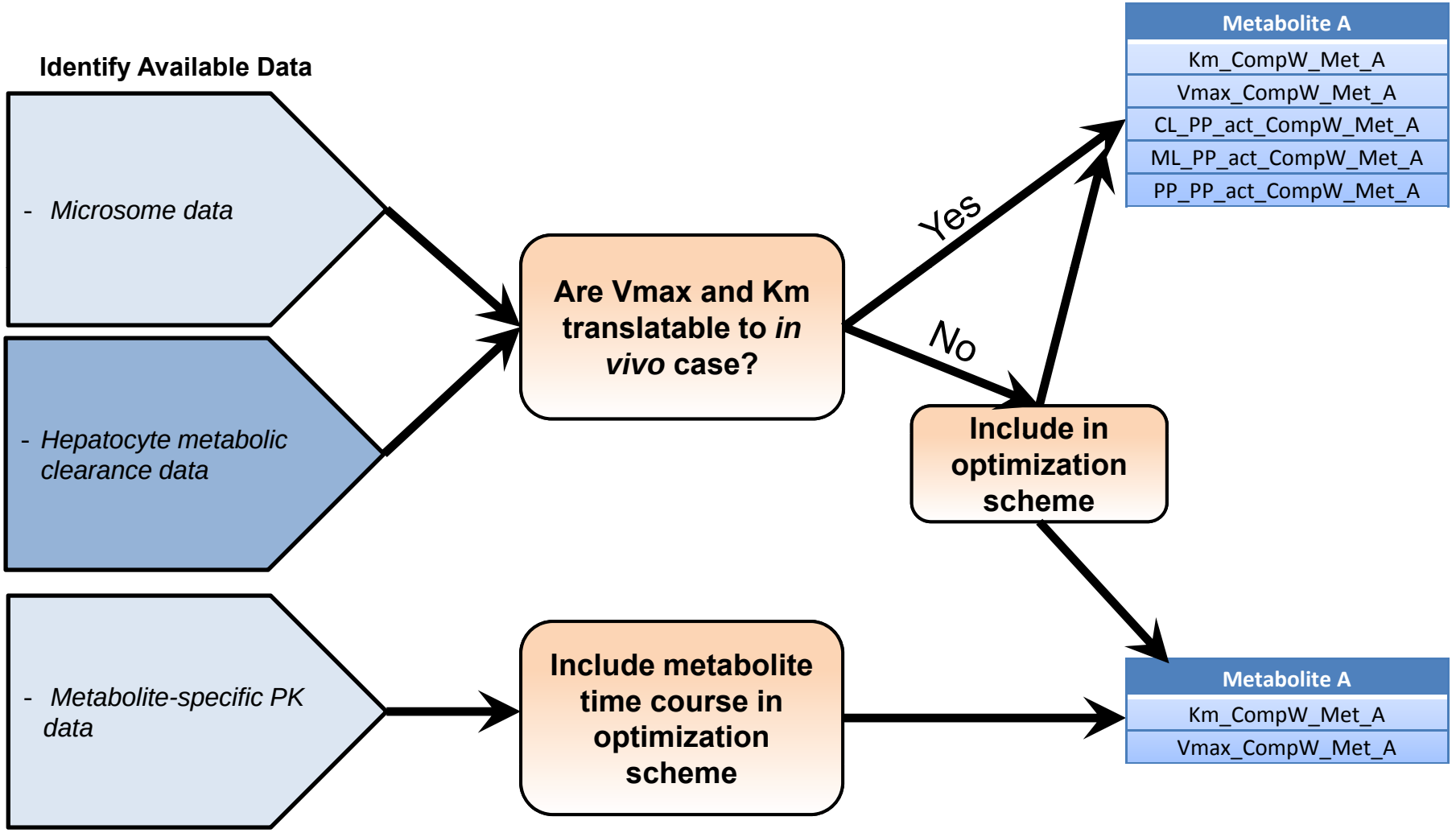
Metabolic clearance of parent compound by specific pathways	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes with co-factors to get quantitative estimate of glucuronidation, sulfation, CYP450 pathway ratios
Mechanism based cytochrome P450 inhibition (drug-drug interaction (DDI) activity)	IC50 value or Ki; reference FDA guidance on DDI's	Industry standard
Potential for active transport in the liver (rate of hepatocyte uptake); transport kinetic information if possible	Hepatocyte suspensions; sandwich cultured (SC) hepatocytes; vesicles	Hepatocyte suspensions
Metabolic clearance of parent compound and coincidental appearance of specific metabolites M	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes to distinguish ph I and ph II pathways
Metabolic clearance of parent compound and coincidental rate of appearance of specific metabolites M	Hepatocytes; liver S9; liver cytosol; liver microsomes	Hepatocytes and microsomes with co-factors to get quantitative estimate of glucuronidation, sulfation, CYP450 pathway ratios
Parent distribution, clearance, and metabolite formation kinetics	*in vivo*	*in vivo*; radiolabel mass balance data preferred

Less confidence

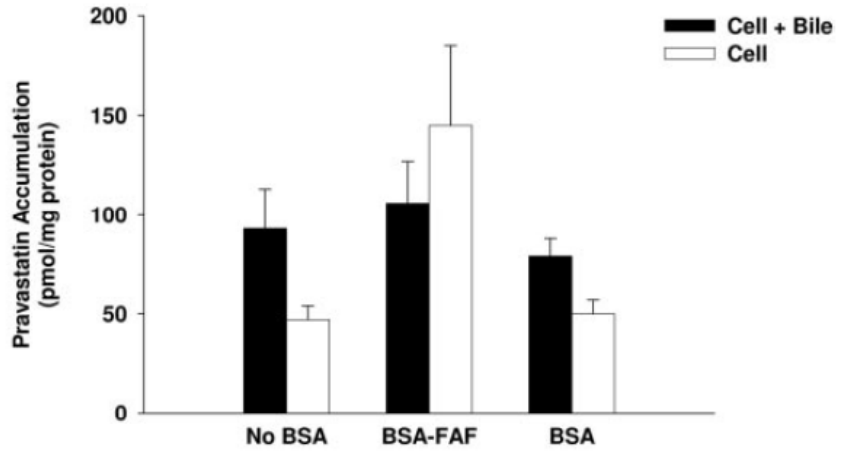
More confidence

Determining Metabolism Parameters for Use in DILIsym®



Relationship of Protein Binding to Toxicologic Activity of Drugs is Complex

- While conceptually appealing to use $f_{u\ Liver}$ as the basis for hepatotoxicity predictions, various observations confound the approach
  - Wolf (2008) shows that cellular pravastatin uptake and biliary clearance are not directly dependent on measured f_u
  - Binding affinity and rate of binding/release can be as important as overall fraction
  - Clearance dynamics are often difficult to replicate with f_u values from *in vitro* experiments (steady state)
  - Compartmentation of drug relative to target may not be captured in estimates of cellular $f_{u\ Liver}$
  - Actual "free" fraction must often be determined empirically from fit to PK data



	No BSA	BSA-FAF	BSA
BEI (%)	$48 \pm 3^{a,b}$	$1 \pm 1^{a,c}$	$37 \pm 3^{b,c}$
Intrinsic $Cl'_{biliary}$ (mL/min/kg)	7.2 ± 1.3^{d}	$0.3 \pm 0.3^{d,e}$	8.5 ± 0.9^{e}
f_u	1^{f}	0.50 ± 0.01^{f}	0.61 ± 0.01^{f}

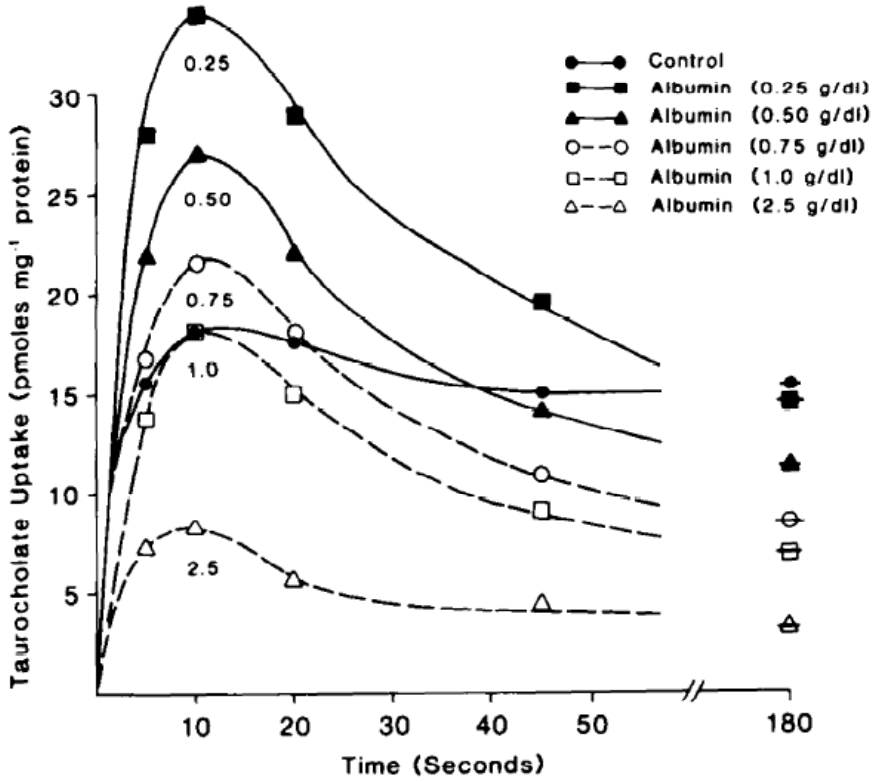
Wolf 2008

Are Toxicity Mechanisms Dependent on "Free" or Total Drug Concentrations?

- Toxicity in hepatocytes could be due to actions of total drug or $f_{u\ Liver}$
  - Currently, toxicity mechanisms are represented within DILIsym® using total drug concentration
- For RNS/ROS and mitochondrial mechanisms, drug protein binding estimates do not affect toxicity parameters and DILIsym® predictions
  - Protein binding is included in *in vitro* environment for hepatocyte studies
    - Assumption: binding in an *in vitro* cell resembles binding *in vivo*
  - Calculation of DILIsym® toxicity parameters requires matching total intracellular concentration to effect *in vitro*
- For transporter inhibition, the answer to this question is key
  - IC50 and K_i values are calculated using vesicle studies where binding proteins may or may not be included
    - Binding to the vesicles themselves may occur
  - What effect does protein binding have on bile acid transport and drug inhibition?
    - Consider binding of both bile acid and drug

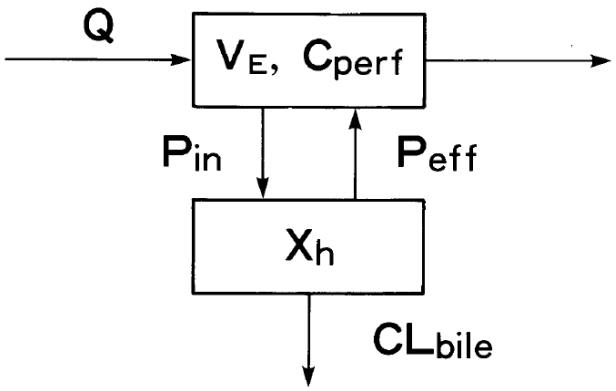
Protein Binding Plays a Role in Bile Acid Transport

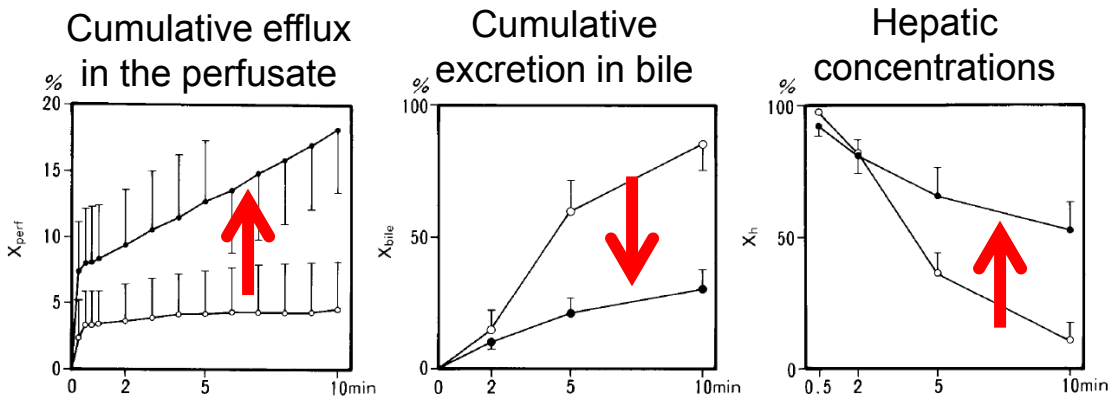
- Blitzer (1985) found that albumin increases both enhanced and inhibited TCA transport via NTCP
  - Dependent on BSA concentration
- Carrier-mediated transport in hepatocytes has been proposed for bile acids
  - Stolz 1993, Alfred 1996
- **Does protein binding enhance or inhibit bile acid transport in cells?**
  - **Does this change as bile acids accumulate in the cell?**



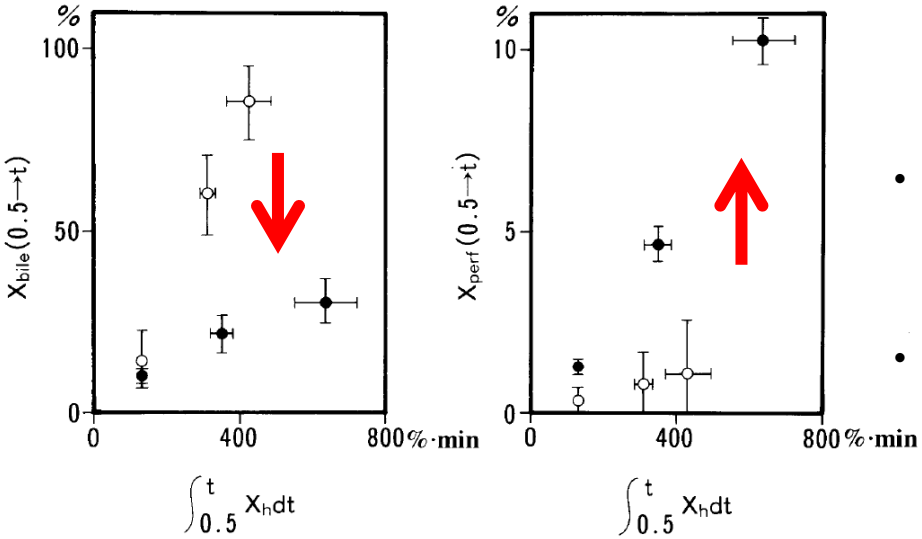
Blitzer 1985

Interaction of Drug with Binding Protein Can Inhibit Bile Acid Transport





Takikawa 1996

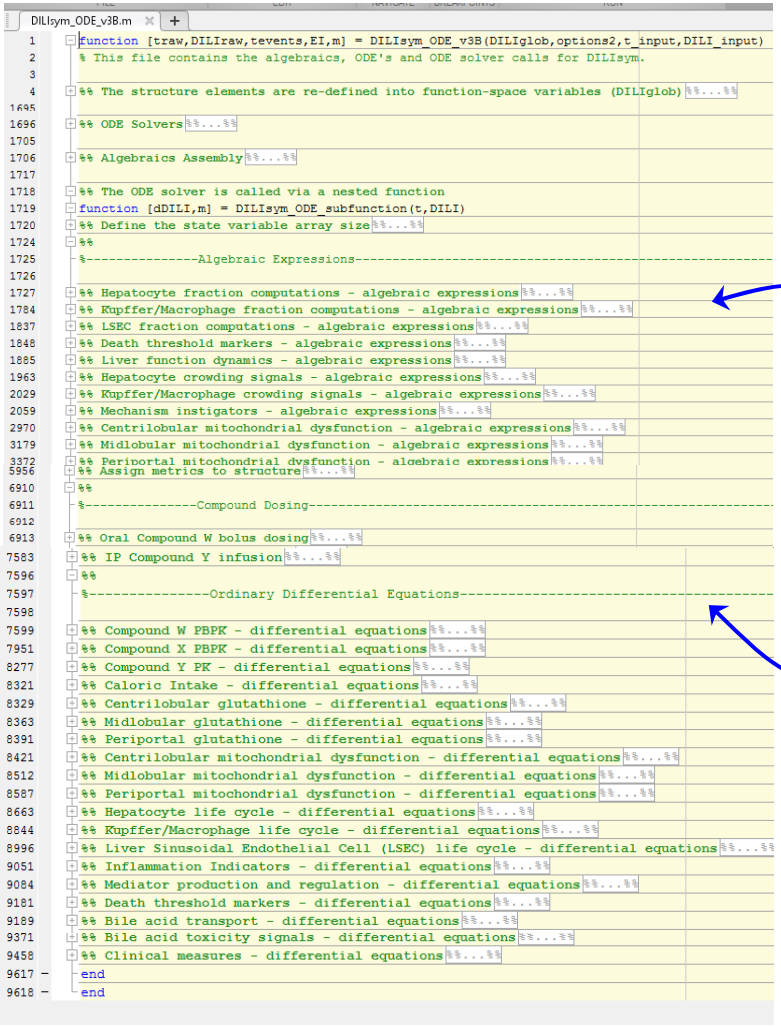


- Indomethacin decreased binding of GCA to cytosolic protein
  - Decreased BA available for biliary excretion
  - Increased BA available for basolateral efflux
- BA bound to cytosolic protein may be the pool for biliary excretion, and unbound BA may be the pool for basolateral efflux or intracellular sequestration
- Interference of drug with binding protein for bile acid (perhaps through its own binding) could inhibit biliary BA flow

Discussion Questions

- Should hepatotoxicity effects in DILIsym® be based upon total drug concentration? Unbound concentration? Effective unbound concentration?
- How should the effect of protein binding on bile acid transport and inhibition be modeled?
  - Should bile acid-protein interactions within cells be considered for future representation in DILIsym®?
  - Should DILIsym® consider adjusting intracellular concentration to account for effect of protein binding on drug's ability to inhibit transporters?
  - Should the ability of drugs to interfere with intracellular trafficking pathways through protein binding be considered for future representation in DILIsym®?
- Are there experiments or literature data that can further elucidate the effect of protein binding on a drug's ability to inhibit bile acid transporters?
- To what extent does protein binding cause a difference between *in vivo* and *in vitro* responses for RNS/ROS and mitochondrial mechanisms?

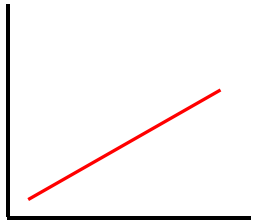
The Primary Equations File within DILIsym® includes Two Main Components



- Filename: *DILIsym_ODE_v3B*
  - Sub-models listed in picture are abbreviated for clarity
- Algebraic expressions are output and rate calculations that often feed into the ordinary differential equations (ODE)
  - e.g. a reaction rate is calculated and fed into a mass balance
- The ODEs are the primary equations solved numerically within MATLAB
- All equations are coded in human-readable form for viewing

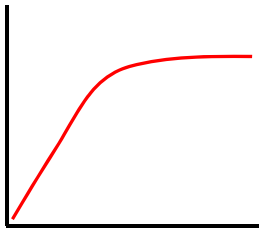
General Equation Structures Often Implemented within DILIsym®

- Linear
  - RNS/ROS production from drug exposure
  - Passive drug transport into organs
  - Non-saturable drug absorption from gut



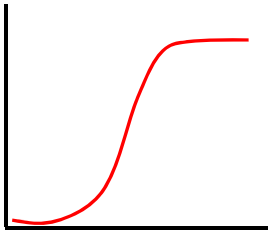
$$Rate = a \cdot C$$

- Michaelis–Menten (saturable)
  - Drug metabolism rates
  - Active drug transport into organs
  - Saturable drug absorption
  - Bile acid transport



$$Rate = \frac{CV_{\max}}{C + K_m}$$

- Hill function (threshold, saturable)
  - Processes with a threshold and saturation are found throughout biology and this structure is commonly used within DILIsym®
  - ROS effects on ATP production
  - ATP depletion causing necrosis
  - Mitochondrial toxicity
  - Innate immune response



$$Rate = \frac{C^n V_{\max}}{C^n + K_m{}^n}$$

Example Equations Implemented within DILIsym® for Specific Pathways or Processes

Competitive transporter inhibition

$$\frac{d[BA]}{dt} = \frac{V_{max}[BA]}{K_m(1+\frac{[I]}{K_i})+[BA]}$$

Noncompetitive transporter inhibition

$$\frac{d[BA]}{dt} = \frac{\frac{V_{max}}{(1+\frac{[I]}{K_i})}[BA]}{K_m+[BA]}$$

Mixed transporter inhibition

$$\frac{d[BA]}{dt} = \frac{V_{max}[BA]}{K_m(1+\frac{[I]}{K_i})+[BA](1+\frac{[I]}{\alpha K_i})}$$

Signal controlling mitochondrial pyruvate utilization based on $\Delta\Psi_m$

$$S_{fb_{H\_Gradient}} = Km_{NegFeedA}{}^n / (Km_{NegFeedA}{}^n + \Delta\Psi m^n)$$

Caspase activating signals, caspase activation, and apoptosis rate

$$CAS\ Input = (fold\ change\ ROS - 1)Scaling\ factor + (fold\ change\ TNF - 1)Scaling\ factor + \ldots$$

$$CAS = \frac{CAS\ Input^n (V\ \max)}{CAS\ Input^n + Km^n}$$

$$Apoptosis\ Rate = CAS(Rate\ const) + Baseline + Direct$$

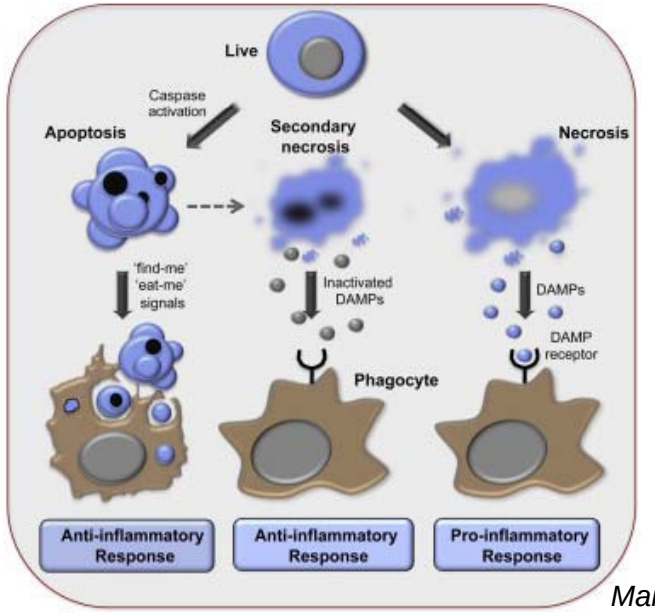
Release of ALT from Apoptotic Hepatocytes

$$\text{ALT Release Rate} = (\text{HC Flux}_{necrosis})(\text{ALT}_{HC}) + (\text{HC Flux}_{apoptosis})(\text{ALT}_{HC})(\text{Secondary Necrosis Indicator})$$

$$\text{Secondary Necrosis Indicator} = f(\text{apoptotic \& necrotic HC bodies})$$

DILIsym® Biomarker Release in Apoptosis and Necrosis

- cK18 added to DILIsym® as a putative biomarker of apoptosis
  - 85% of HC K18 is cleaved on apoptosis and released (Kramer 2004)
- Published data are equivocal on whether ALT is released on apoptosis *(e.g., Kronenberger 2000, 2005, Oberhammer 1996, Lawson 1999, Antoine 2009, Canbakan 2010, Calabrese 2000)*
- Biomarker design allows apoptosis-driven release of non-cK18 biomarkers when a threshold for apoptotic cells is exceeded
  - Reflects concept that apoptotic cells can exceed the removal capacity of phagocytes, leading to secondary necrosis



Martin 2012

Release of ALT from Apoptotic Hepatocytes

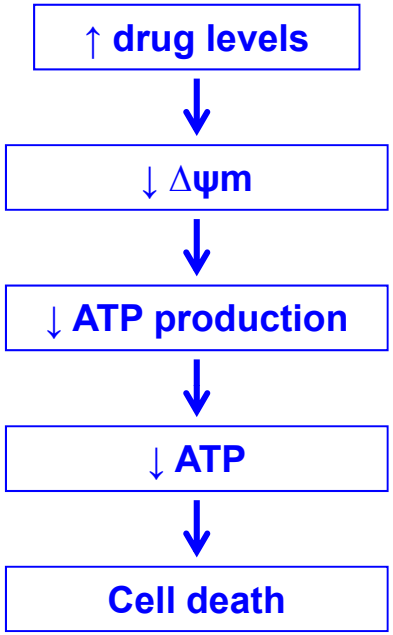
$$\text{ALT Release Rate} = (\text{HC Flux}_{\text{necrosis}})(\text{ALT}_{\text{HC}}) + (\text{HC Flux}_{\text{apoptosis}})(\text{ALT}_{\text{HC}})(\text{Secondary Necrosis Indicator})$$

$$\text{Secondary Necrosis Indicator} = f(\text{apoptotic \& necrotic HC bodies})$$

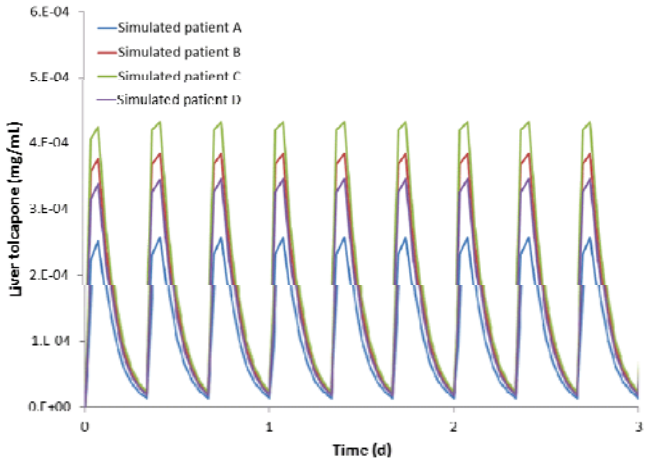
Apoptosis	**Inefficient clearance**	**Necrosis / Necro-apoptosis**
cK18	**(threshold)**	**(no threshold)**
		HMGB1
	FL-K18	FL-K18
	ALT	ALT
	AST	AST
	SDH	SDH
	Arg-1	Arg-1
	miR-122	miR-122

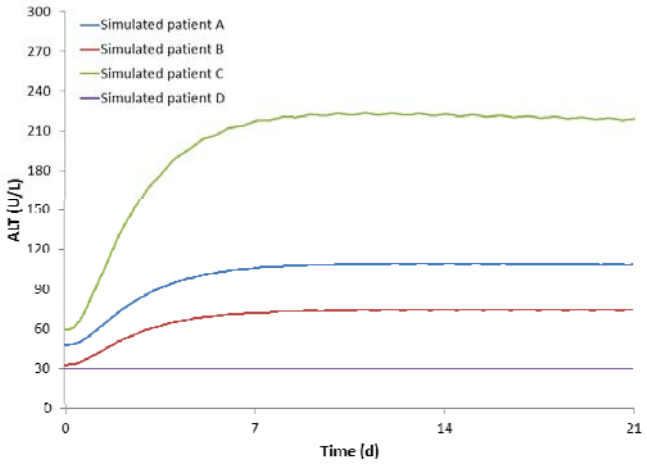
Delayed DILI Onset not Predicted for ROS and Mitochondria Toxicity Mechanisms in DILIsym®

- Mechanistic connections between drug or metabolite and hepatocyte drive toxicity response in DILIsym®
  - Based on current understanding
- Timing of toxicity response is determined by time required for intracellular compound (or metabolite) to initiate mechanistic changes
  - Injury presentation often predicted to be coincident with exposure steady state
  - Within 3 days of t.i.d. dosing with tolcapone
- Elevated liver signals frequently observed in patients later than predicted in DILIsym®
  - The observed median time to ALT 3X ULN was 81 d in patients for tolcapone, with range 30-120 d (Olanow 2007)
  - The predicted time for ALT to reach peak levels in DILIsym® was 7 d with tolcapone
- Possible explanations for difference between observed and predicted timing of injury include
  - Compartmental drug accumulation (e.g. perhexilene)
  - Heteroplasmy threshold (Boelsterli 2007)
  - Accumulating adducts
  - 2nd hit



HUMANS

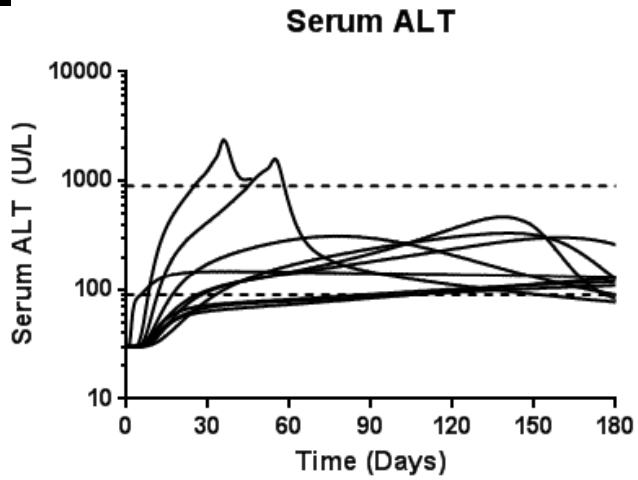




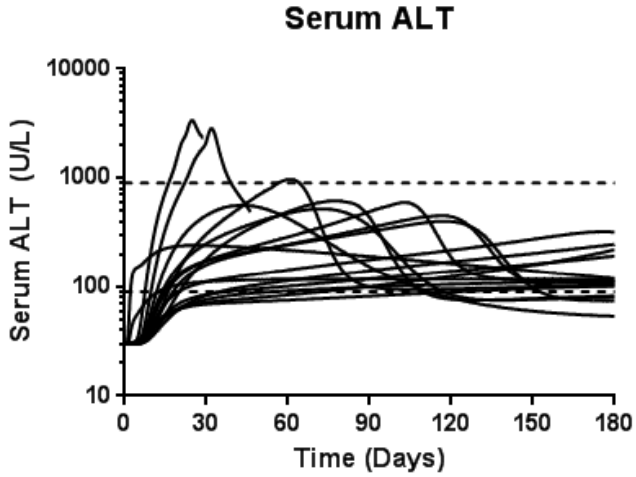
DILIsym® Predicts Delayed Presentation of Troglitazone-Mediated Hepatotoxicity

HUMANS

Toglitazone 400 mg/day



Troglitazone 600 mg/day



	Simulations		Clinical Trials
	TGZ 400 mg	TGZ 600 mg	TGZ 200 – 600 mg
Time to peak ALT (Days)	118 ± 61	111 ± 61	147 ± 86

- The delayed ALT elevations in DILIsym® were driven by a delayed build-up of toxic bile acids in hepatocytes
  - FXR-mediated feedback regulation of bile acid synthesis/transport initially delayed bile acid accumulation until it could no longer compensate
  - Troglitazone and troglitazone sulfate competitively inhibits BSEP; as hepatic bile acids increase and outcompete the inhibitor, the rate of bile acid accumulation slows down

Yang et al. CPT (in press)