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Please note: this presentation, including questions from the audience, is being recorded

DILIsym Review Session 23:

Protein Binding in DILIsym

May 17, 2018

Grant Generaux

DILIsym Review Session Agenda

- Review of protein binding in DILIsym's PBPK representation
- Review of protein binding for DILIsym's hepatotoxicity mechanisms and bilirubin representation
- Free/Bound Focus Group background and update

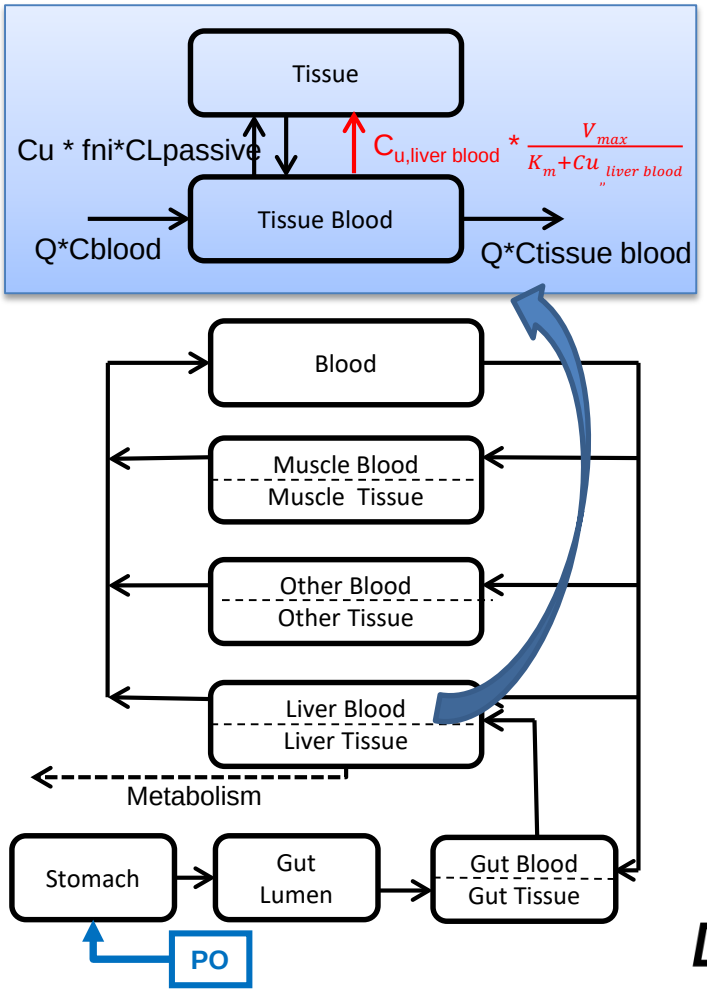
Protein Binding in the DILIsym® PBPK Sub-Model

- Drug disposition in the PBPK sub-model is based on the unbound concentration
  - Hepatic/intestinal metabolism and transport
    - Active transport and passive diffusion
  - Renal and biliary excretion
- Fraction unbound in plasma is a DILIsym user input
  - Linear and non-linear (concentration-dependent) plasma protein binding can be represented
- Fraction unbound in tissue is calculated by DILIsym
  - The fraction unbound in liver and enterocytes can be defined by the user

Perfusion- and Permeability-limited Distribution Represented for All Tissues Using a Two-compartment Tissue Model

fni: fraction non-ionized

Active uptake transporter represented only in the liver

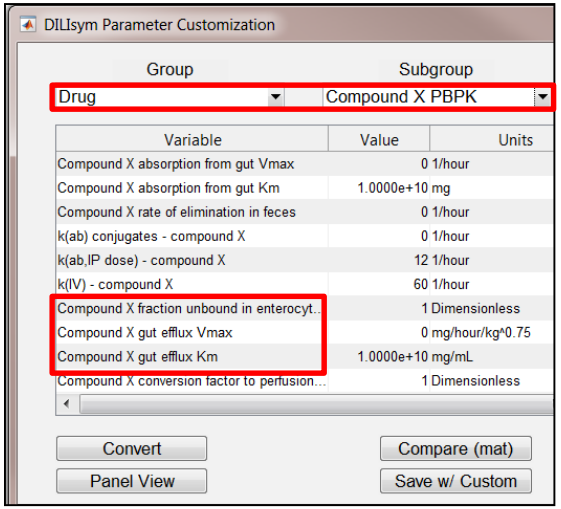


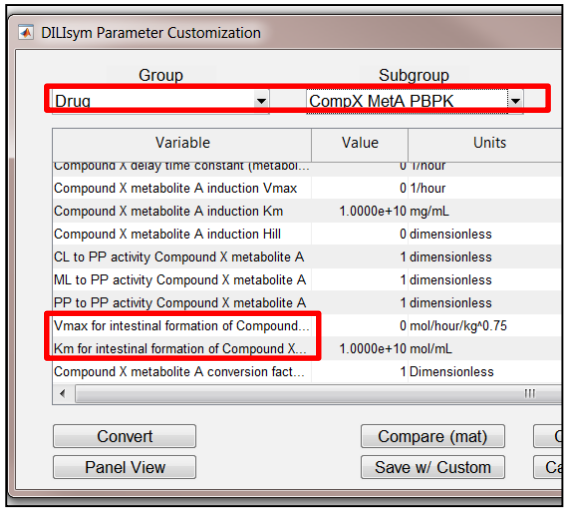
- Perfusion-limited distribution if CLpassive >> Q (default)
  - Instant mixing of tissue and tissue blood; reach equilibrium quickly
  - Extent of tissue distribution will be determined by fu_P and fu_T (calculated using tissue:blood ratio), pKa, compound type (acid/base)
  - User input: fu_P, B:P, Tissue:Blood partition coefficients, compound type (acid/base), pKa

- For permeability-limited distribution, CLpassive of each compound can be optimized or calculated from *in vitro* permeability data
  - Only unbound, non-ionized drugs can undergo passive diffusion; frac non-ionized calculated by DILIsym using compound type (acid/base) and pKa values
  - CLpassive calculated by DILIsym using *in vitro* permeability and the tissue surface area
  - User input: compound type (acid/base), pKa, *in vitro* permeability, transporter Km/Vmax if a substrate of hepatic uptake transporters

Intestinal Metabolism and Transport is Driven by Unbound Gut Concentration

- In DILIsym intestinal metabolism and transport are available for Compound X and W
  - Based on the unbound gut concentration
  - The user can define “Compound X fraction unbound in enterocyte”
- Stable metabolites (metabolite A and B) can be generated by gut metabolism
  - Saturable process (K_m and V_{max})
  - Generated metabolites enter into liver tissue and are combined with liver-generated metabolites
- Efflux of parent compounds from gut tissue to intestinal lumen represented
  - Saturable process (K_m and V_{max})





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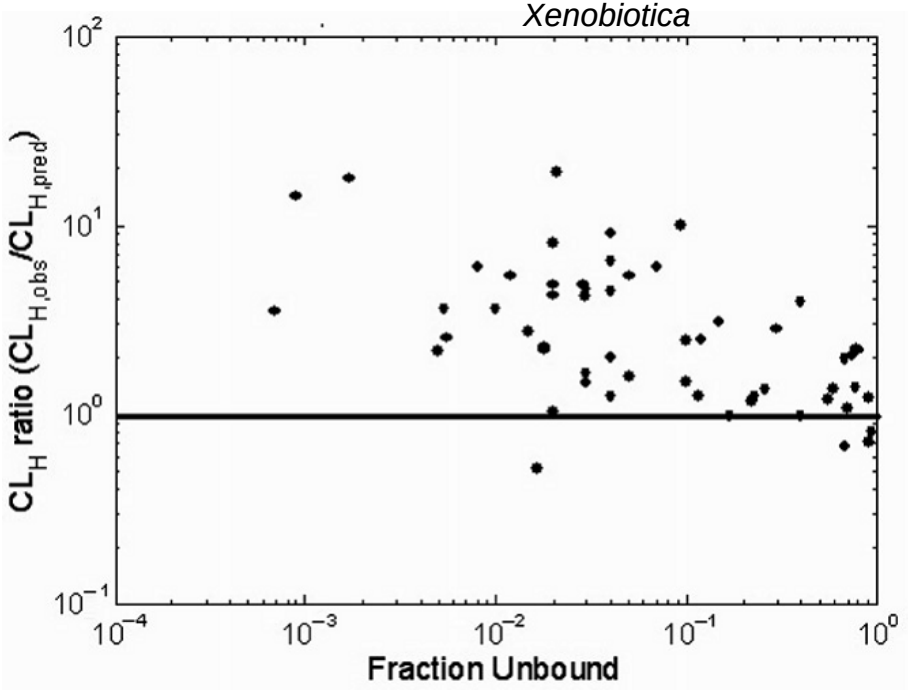
Determination of Fraction Unbound Requires Both *In Vitro* Data and Optimization

- 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
  - Poulin (2015) suggests that albumin may actually facilitate transport into hepatocytes; suggests a correlation for calculation of $f_{u,adjusted}$
- *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

Baker (2007)
Xenobiotica



Preclinical and Clinical Data

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Determining Parameter Values for Tissue Distribution and Protein Binding

Data Available for Compound

- *Log P*
- *pKa*
- *Fraction unbound (blood)*
- *B:P ratio*

- *Absorption – IV dosing*
- *Organ partition coefficients and fractions unbound*
- *Renal clearance*

- $$Kpu = \frac{C_t}{C_{u,p}}$$

Ratio of drug concentration in tissue to drug concentration unbound in plasma

- $$\frac{C_{tissue}}{C_{blood}} = \frac{C_{tissue}}{C_{plasma,free}} \times \frac{C_{plasma,free}}{C_{plasma}} \times \frac{C_{plasma}}{C_{blood}}$$

Ratio of drug concentration in tissue to drug concentration in blood

$$Kpu \times f_u \times \frac{1}{B:P}$$

- $$\frac{C_{tissue}}{C_{blood}} = Kpu \times f_u \times \frac{1}{B:P}$$

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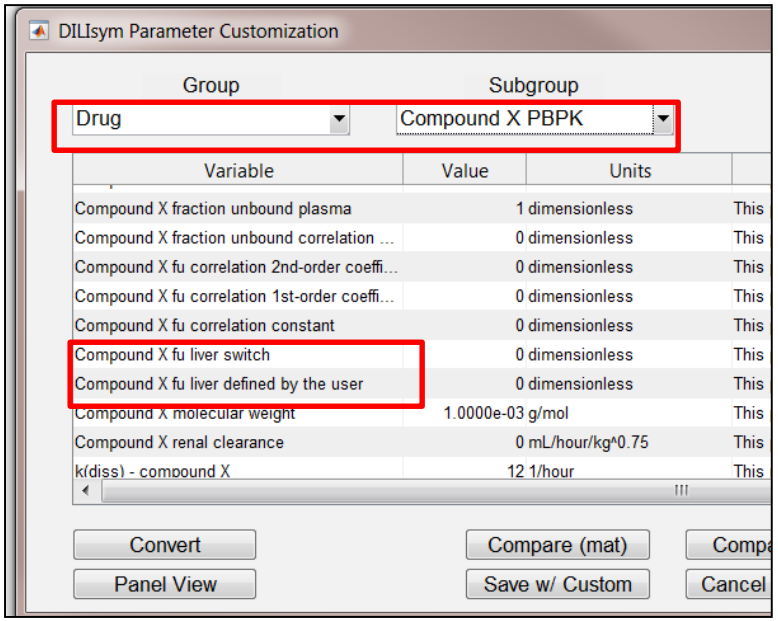
Fraction Unbound in Tissue Is Calculated by DILIsym or Can Be Defined by the User

- DILIsym automatically calculates tissue fraction unbound for liver, gut, muscle, and other tissue

- Alternatively, the user can define the unbound fraction in the liver using the “Compound (X) fu liver switch”
  - If the switch is set to 1, “Compound (X) fu liver defined by the user” will be used in the PBPK sub-model



Fraction Unbound in Tissue Calculated by DILIsym

- Unless the user turns on the “Compound (X) fu liver switch”, DILIsym calculates tissue fraction unbound for liver, gut, muscle, and other tissue automatically
- In case of passive diffusion, the unbound tissue concentration is equal to the unbound plasma concentration
  - $f_{u,tissue}$ is calculated from partition coefficients and the blood:plasma ratio

$$f_{u,tissue} = \frac{f_{u,plasma}}{\left(\frac{C_{tissue}}{C_{blood}}\right) \times B:P}$$

- In case of transporter-mediated liver uptake, the unbound liver concentration is not in equilibrium with the unbound plasma concentration
  - An empirical equation is used to estimate $f_{u,liver}$ (Poulin and Theil 2000)

$$f_{u,cell} = \frac{1}{1 + \left(\frac{1 - f_{u,p}}{f_{u,p}} \cdot C_{m,tissue}\right)}$$

Cm,tissue: relative albumin conc in the liver compared to plasma (often assumed to be 0.5)

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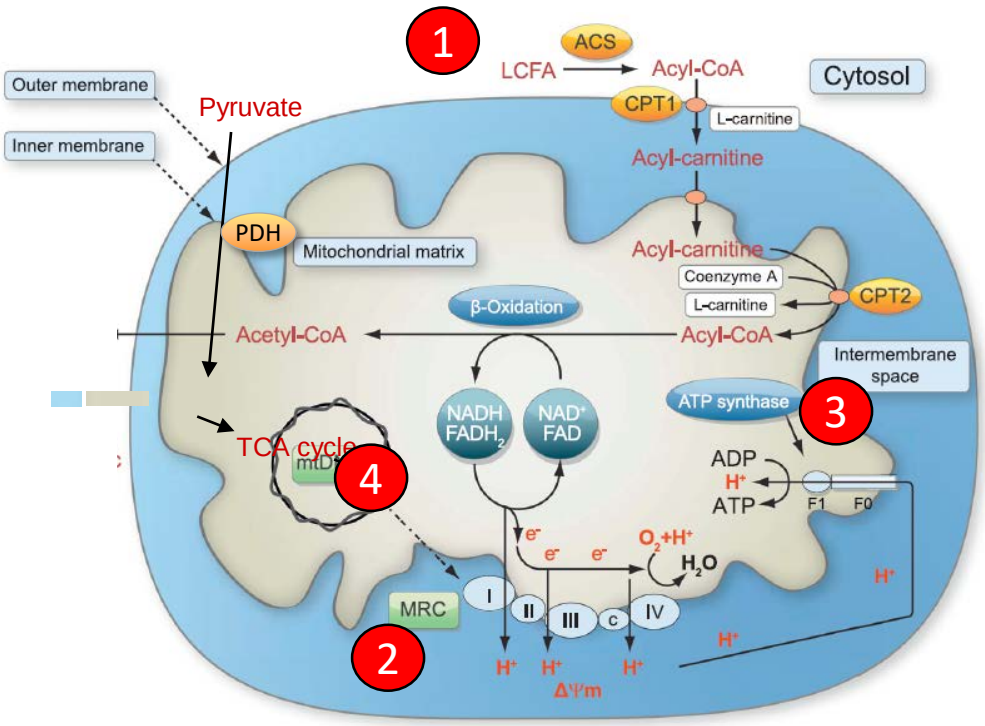
Relevant Biophases for Mitochondrial Dysfunction

1. Outer membrane of mitochondria contains *porins* which allow free diffusion of molecules up to 5kD.
2. Concentrations in the intermembrane space closely approximate the cytosol due to the porins and decreased permeability of the inner membrane.
3. Inner membrane of mitochondria is particularly impermeable to molecules,
4. partially due to the presence of *cardiolipin*



For ETC inhibition, ATP synthase inhibition, and uncoupling the inner membrane appears to be the most relevant biophase

Adapted from Begriche 2011

Toxicity Mechanisms in DILIsym are Represented Using Total Drug Concentration

- Toxicity in hepatocytes could be due to actions of total drug or $C_{u\ Liver}$
  - 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
  - The *in vitro* environment for HepG2 and hepatocyte studies can include protein or no, depending on the optimization of the assay conditions
  - Calculation of DILIsym toxicity parameters requires matching measured total intracellular concentration to effect *in vitro*
    - Assumes that binding in an *in vitro* cell resembles binding *in vivo*

- For bile acid transport, the situation is more complicated
  - IC50 and K_i values are typically calculated using vesicle studies where binding proteins are not 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

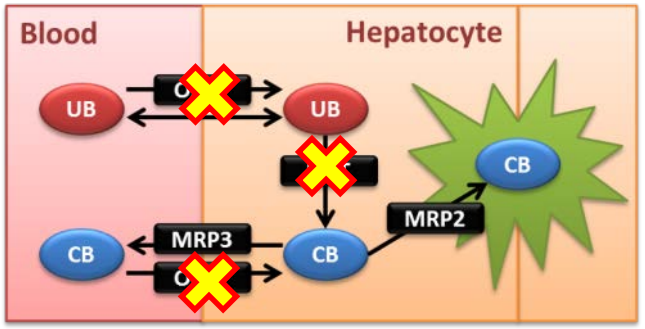
Bilirubin Submodel Utilizes Unbound Concentrations

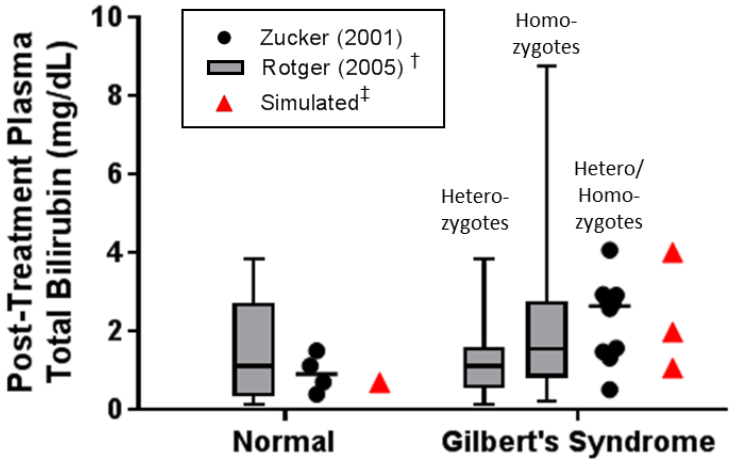
- Indinavir and nelfinavir inhibit UGT1A1 and OATP1B1

Enz/Transporter	Indinavir	Nelfinavir
OATP1B1 IC_{50} (µM)	4.1	2
UGT1A1 IC_{50} (µM)	6.8	4.8
MRP2 IC_{50} (µM)	>100	>100

- Unconjugated hyperbilirubinemia is observed in 6-25% of HIV patients receiving indinavir, but nelfinavir is not associated with hyperbilirubinemia
  - Indinavir-mediated hyperbilirubinemia is more pronounced in individuals possessing GS alleles

- DILIsym accurately predicts indinavir-mediated unconjugated hyperbilirubinemia (shown on right) and minimal impact of nelfinavir on serum bilirubin (not shown)
  - Potentially due to greater unbound plasma/liver concentrations of indinavir compared to nelfinavir
  - Although systemic exposure is comparable, nelfinavir is highly bound to protein





†Indinavir and atazanavir data combined. Indinavir and atazanavir increased bilirubin by 0.46 and 0.87 mg/dL, respectively.
‡ 800 mg indinavir tid for 1 month

Clinical Data and Simulation Results

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- Review of protein binding in DILIsym's PBPK representation
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DILIsym Free/Bound Focus Group

Objective of Focus Group: Review and evaluate the use of total vs. free drug as a driver of toxicity in DILIsym to identify a strategic path forward.

Mechanism	Actual Biophase	Representative Concentration in DILIsym		
		Total Liver	Free Liver	*In vitro* measurement
Canalicular efflux	Apical hepatocyte membrane		✓	**Active:** $[Cell] * fu_{cell}$ **Passive:** [Media]$* fu_{inc}$
Mitochondrial uncoupling	Inner mitochondrial membrane		✓	
ETC inhibition	Mitochondrial matrix		✓	
mtDNA depletion	Mitochondrial matrix		✓	
Reactive-metabolite mediated	Hepatocyte proteins	✓		

Bosentan and AMG009 Toxicity Is Recapitulated With Total But Not With Free Drug

- Using free drug as the biophase for BSEP inhibition did not lead to any prediction of toxicity
  - Liver bile acid levels did not change
- Reason why total drug produces better predictions remains unclear
  - Assay system nonspecific binding may contribute to K_i measurement
    - Literature on nonspecific binding in vesicle systems is sparse
  - Drug protein binding may interfere with bile acid intracellular trafficking
  - Protein-drug-bile acid interactions are complex; weak protein binding may enable some "bound" drug to inhibit transporters

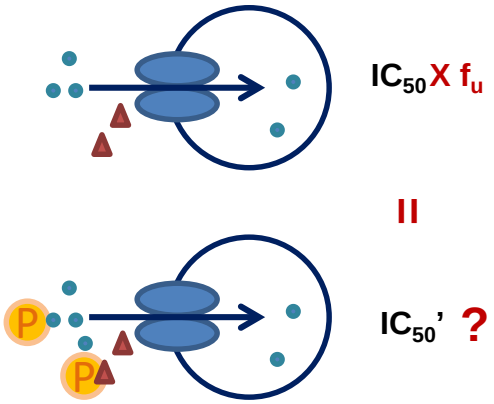
Drug	Biophase	ALT > 3x ULN
Bosentan 500 mg BID	Total	24/285 (8.4%)
Bosentan 500 mg BID	Free	0/285
Clinical Observation	-	**8-18%**

Drug	Biophase	ALT > 3x ULN
AMG-009 100 mg QD	Total	112/285 (38.3%)
AMG-009 100 mg QD	Free	0/285
Clinical Observation	-	**12.5%**

Simulation Results and Clinical Data

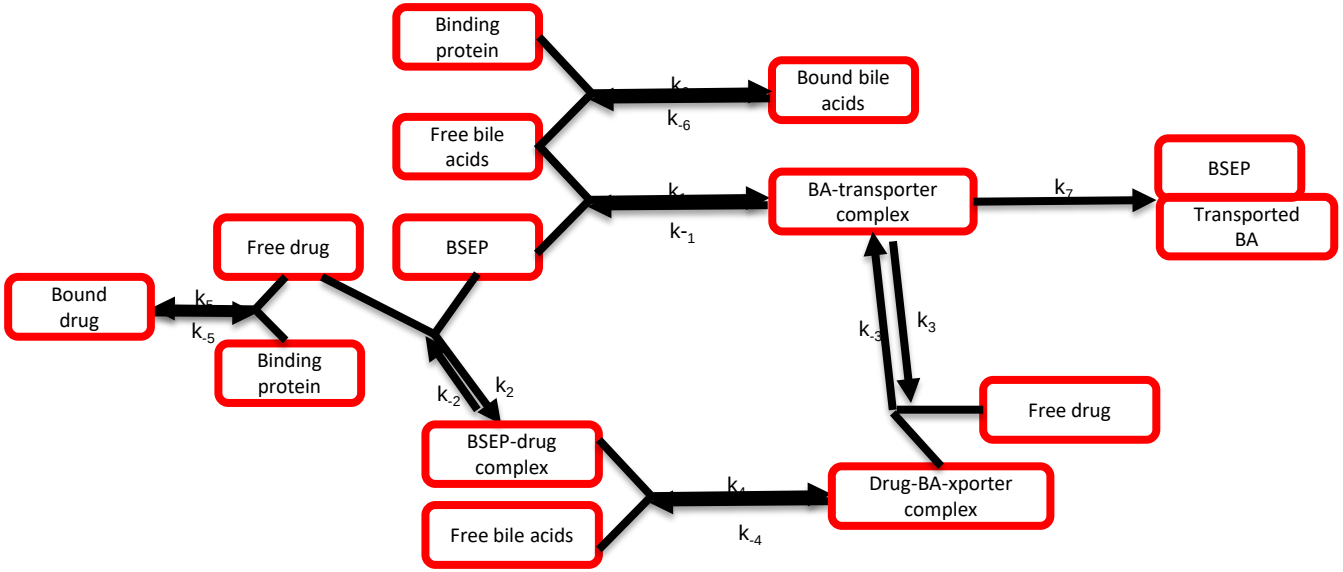
In Vitro Assays to Determine Bile Acid Transporter Inhibition

- Correction of non-specific binding in IVIVE
  - Correcting for non-specific binding in the *in vitro* microsomal assays improves the accuracy of *in vivo* metabolic clearance prediction
  - In *in vitro* vesicular assays, non-specific binding to the vesicles and/or apparatus may occur
  - Will it improve hepatotoxicity prediction if we correct for non-specific binding in the *in vitro* transporter assays?
- The effect of protein binding on a drug's ability to inhibit bile acid transporters
  - Certain drugs with high plasma protein binding (e.g., kinase inhibitors) have high V_d and short half-life indicating non-restrictive binding; need to consider K_{on}/K_{off}
  - Transporter-induced protein binding shift observed in transporter assays where K_m and IC_{50} values were compared in the absence and presence of protein (Pouline 2015, Baik 2015)
  - Does the current practice (i.e., transporter kinetics are measured without protein and then adjusted by unbound fraction) under-predict a drugs inhibitory effects on bile acid transporters in vivo?



- Complex drug-bile acid interaction in the intracellular environment
  - Intracellular binding, trafficking, sequestration of bile acids and drugs
  - Experimentation not straightforward

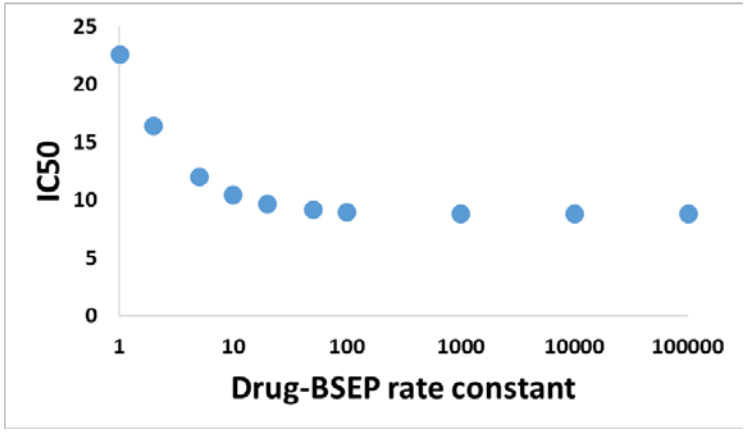
Model of Protein Binding in Bile Acid Biochemistry Being Developed

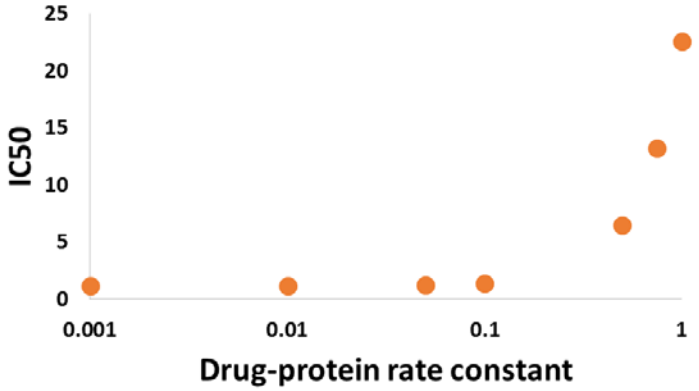


- Model of protein-drug-bile acid interactions under construction and testing
  - Separate from DILIsym model; potential for inclusion of these effects within DILIsym in the future
  - Initial assumption: only free bile acids and drug can interact with transporters
  - Both free drug and free bile acids draw from same pool of binding protein
- Exploration of model can elucidate potential role for unknowns in protein binding biochemistry
  - Overall concentration of binding protein (especially as bile acids build up)
  - Relative affinity of drug for protein binding and transporters
- Further complexity can be introduced as exploration warrants

Apparent IC_{50} Depends on Affinity of Drug for BSEP and Binding Protein

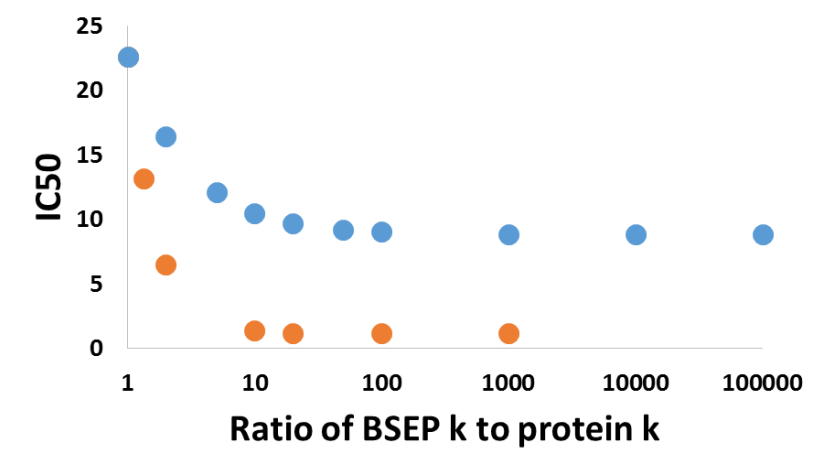
- Simulations in protein binding-bile acid mini-model run varying rate constants for drug-BSEP binding and drug-protein binding with *in vivo*-like physiological conditions
  - In vitro (protein-free) K_i = 1 μM
  - Protein concentration = 2320 μM; this is likely high
  - BSEP concentration = 2.89 x 10^{-4} μM
  - K_m = 18 μM
  - Drug and bile acid both 5% unbound
- Varying rate constant for drug interactions changes apparent IC_{50} of the system
  - When rate constants are equal, apparent IC_{50} is ~22 μM
    - About what one would expect from free drug hypothesis (1/0.05 = 20)
  - Higher rate constant for drug-BSEP interaction and lower rate constant for drug-binding protein interaction both change apparent IC_{50}
- Curves are similar when BA is present at physiological and *in vitro*-like conditions





Hysteresis Observed for Ratio of Drug-Protein Interactions

- Relative affinity of drug-protein interactions hypothesized to change apparent IC_{50} in bile acid inhibition
  - High affinity of drug for BSEP relative to binding protein would lead system away from “free drug” hypothesis
- While this appears to be the case, there appears to be a difference between when drug-protein interactions are changed vs. when drug-BSEP interactions are changed
  - Unclear why this would be the case
    - Error in model assumptions?
    - Emergent behavior?



Simulation Results

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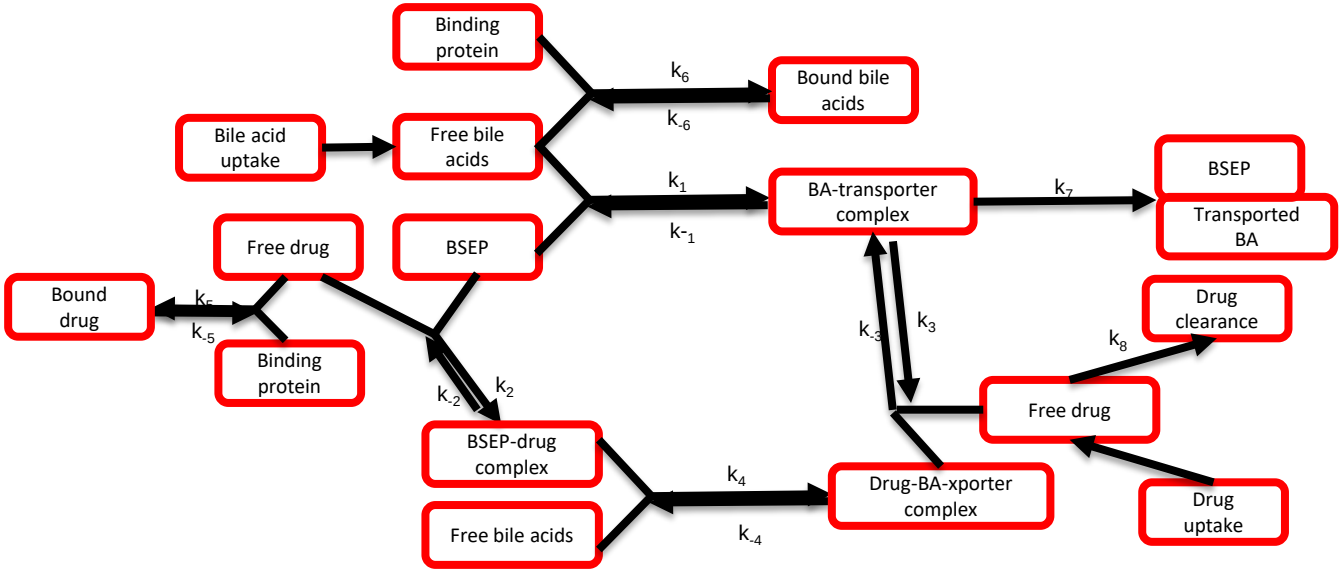
Dynamics of Bile Acid and Drug Added to Protein Binding Mini-Model



- Bile acid uptake and drug uptake/clearance added in order to mimic dynamics of *in vivo* system
  - Bile acid uptake optimized to maintain steady state of total BA concentration in cell
  - Meal signals add uptake in order to increase total intracellular BA to a maximum of 2-3-fold above the baseline value; this is consistent with representation in DILIsym
- System can track bile acid buildup over time
- Initial simulations suggest affinity matters here too, though drug-protein affinity also affects drug clearance behavior

Protein Binding Model Can Be Adapted to Represent More Complex Behavior

- "Chaperoning" – where bile acid transport is facilitated by protein binding - can be represented
  - This has been investigated briefly within the mini-model
  - Under chaperoning conditions, highly-bound drug may actually disrupt BA transport more than less highly-bound drug
    - This is suggested as a contributor to indomethacin-induced DILI by Takikawa 1996, though others blame disruption of phospholipid-bile acid interactions
- Binding to multiple intracellular protein pools (i.e. drug and bile acids not necessarily competing for same binding proteins) can be added
- Potential saturation of/competition for intracellular L-FABP sites by intracellular free fatty acids under NASH/NAFLD conditions
  - L-FABP is the main bile acid binding protein and is present at concentrations of 100-400 μM (Atshaves 2010, Favretto 2015)
  - Initial simulations suggest that increased drug affinity for binding protein increases amount of free bile acids even without chaperoning present

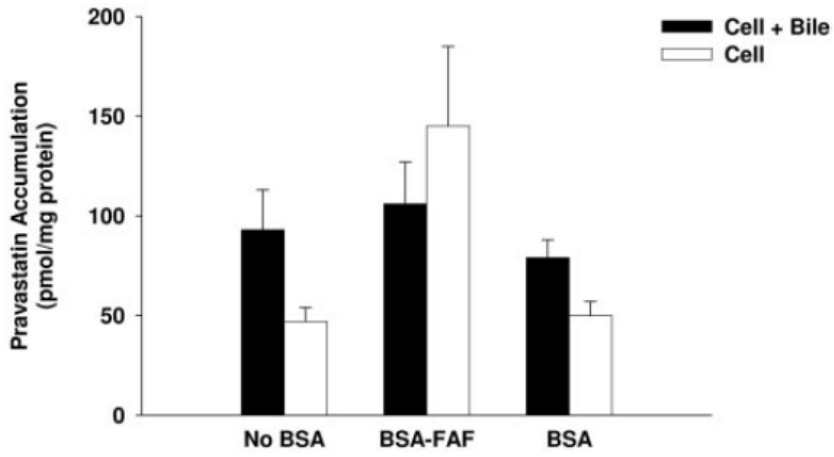
Summary

- Unbound concentrations drive plasma and tissue exposure in DILIsym's PBPK representation
- Toxicity mechanisms in DILIsym are represented using total drug concentration
- Bile acid-drug binding "mini-model" may aid in elucidating a path toward representing bile acid-driven toxicity based on free-drug concentrations

Backup slides

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 ± 3[a,b]	1 ± 1[a,c]	37 ± 3[b,c]
Intrinsic $Cl'_{biliary}$ (mL/min/kg)	7.2 ± 1.3[d]	0.3 ± 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

Preclinical Data

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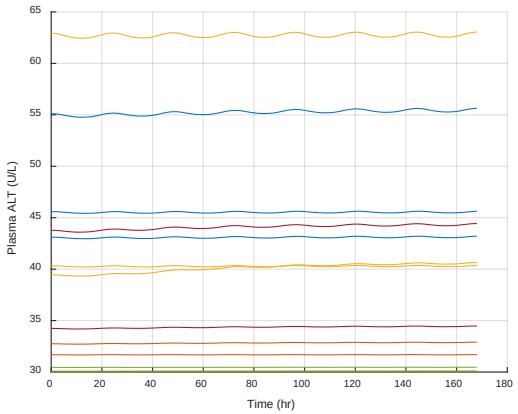
Tolcapone ALT Increases Are Recapitulated With Total But Not With Free Drug

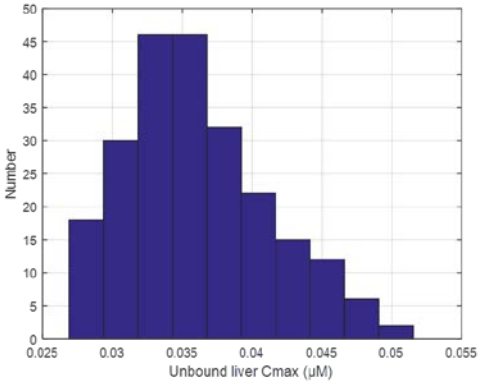
- Using free liver as the biophase for mito uncoupling did not lead to plasma ALT changes.

Plasma ALT

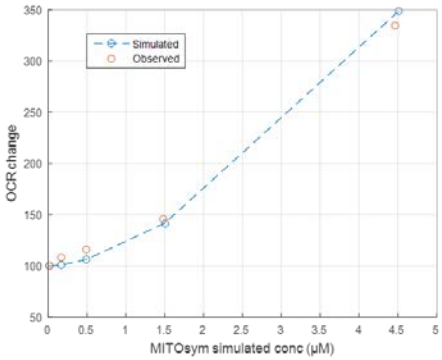
Free



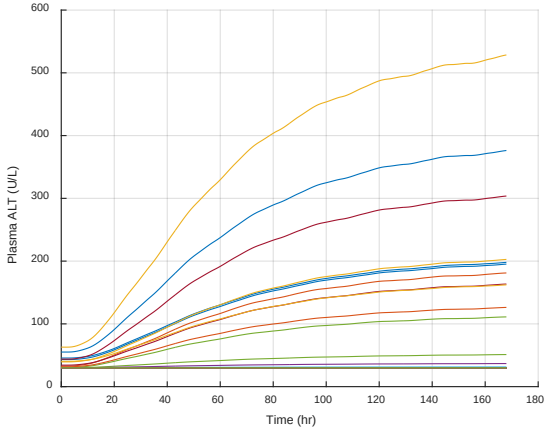
Human_mito_BA_v3A_6' SimPops



MITOsym Free Intra. HepG2 Conc-response



Total



Simulation Results

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Further Exploration of Tolcapone Free Liver Simulations is Proceeding

- Several potential lines of inquiry for understanding initial tolcapone free results
  - Free intracellular HepG2 concentrations were predicted using DILIsym's Kpu and fu_L values – plays key role in MITOsym calibration
    - Kpu assumes equilibrium, but OCR was measured 20 min after addition of tolcapone – enough time to equilibrate across membrane?
  - Recently released MembranePlus™ version 2 has the potential to give us more insight into the dynamics of intracellular concentrations in our in vitro systems.
  - Consider collecting additional tolcapone exposure data in HepG2 cells (e.g., free media concentrations) to reduce this uncertainty.