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DILIsym User Training – Representing Reactive Metabolites in DILIsym

DILIsym Development Team

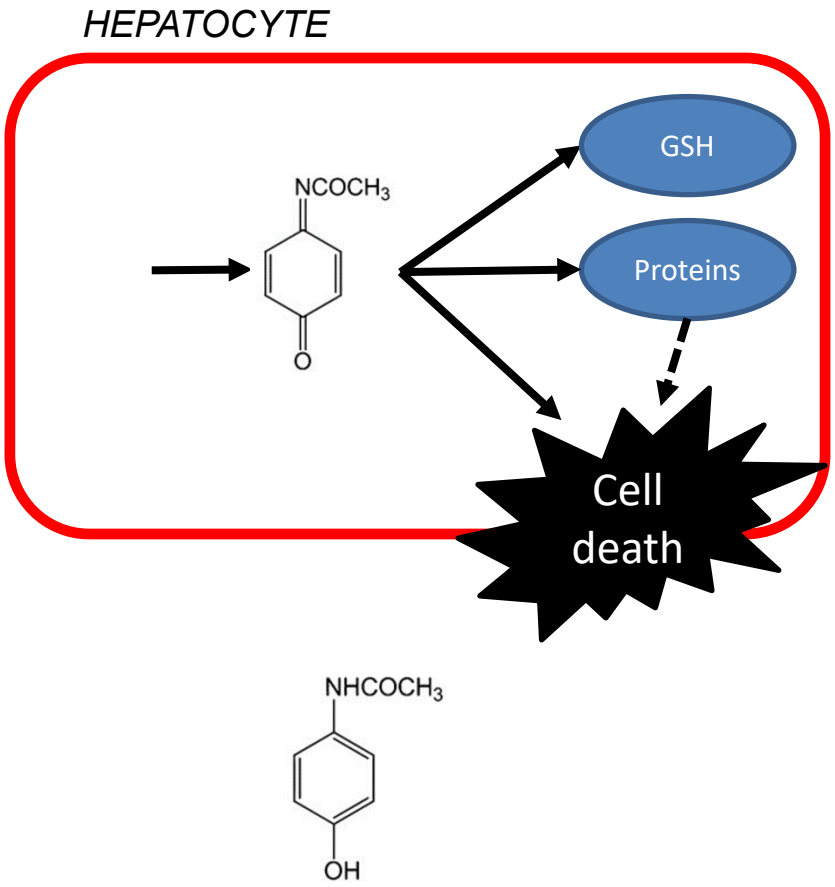
Goal for This Training Session

Participants should understand the following general concepts:

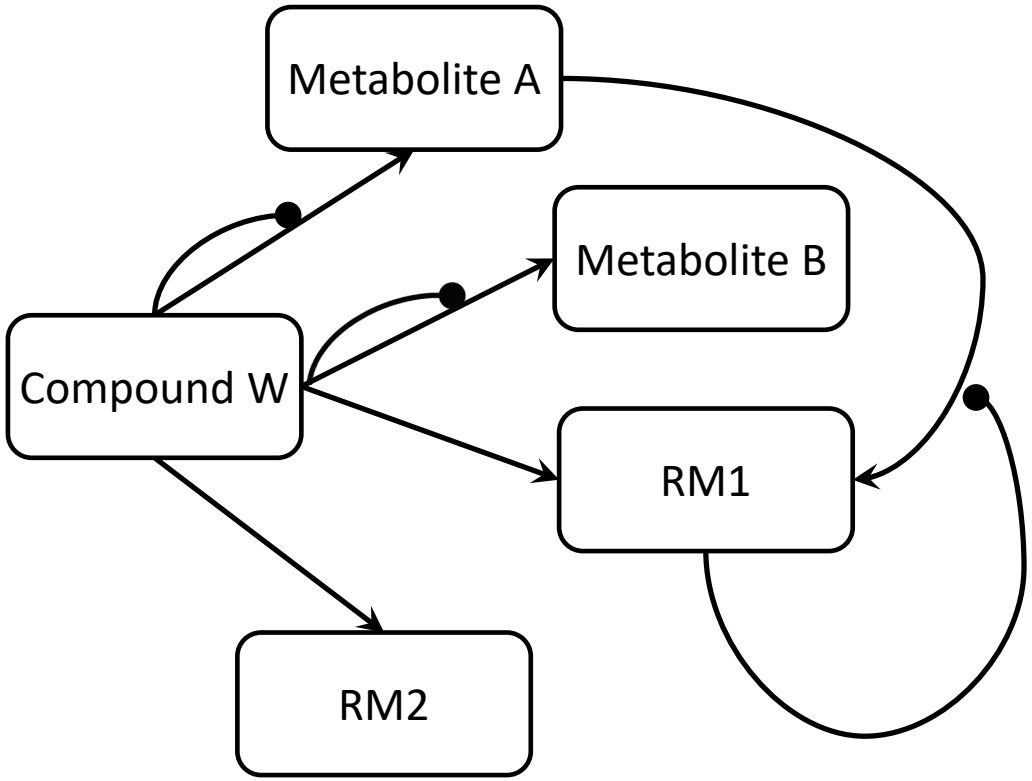
- Background and DILIsym design information for reactive metabolite representation within DILIsym

Reactive Metabolite-Mediated DILI

- Reactive metabolites (RM) are extremely labile metabolites generated in the liver that can covalently bind to cellular proteins or cellular antioxidants
- Ability to cause toxicity determined by amount of reactive metabolite generated and ability of the reactive metabolite to cause cellular stress
  - Reactive metabolites often, but not always, cause oxidative stress
  - Reactive metabolites themselves or adducts to cellular proteins can cause toxicity
- Examples of drugs that have reactive metabolites
  - Acetaminophen
  - Furosemide
  - Cocaine
  - Methapyrilene



Compound Metabolism Representation in DILIsym Includes Two Reactive Metabolites



- DILIsym metabolic scaffold involves four potential metabolic pathways
  - Two stable metabolites and two reactive metabolites
- Reactive metabolites can be generated from parent compound or from Metabolite A
- Metabolism to RM values are difficult to calculate directly
  - Usually need information on RM fate (GSH depletion, protein adducts) from microsomal data in order to properly determine RM metabolism

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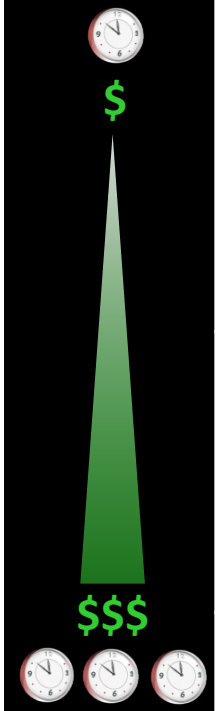
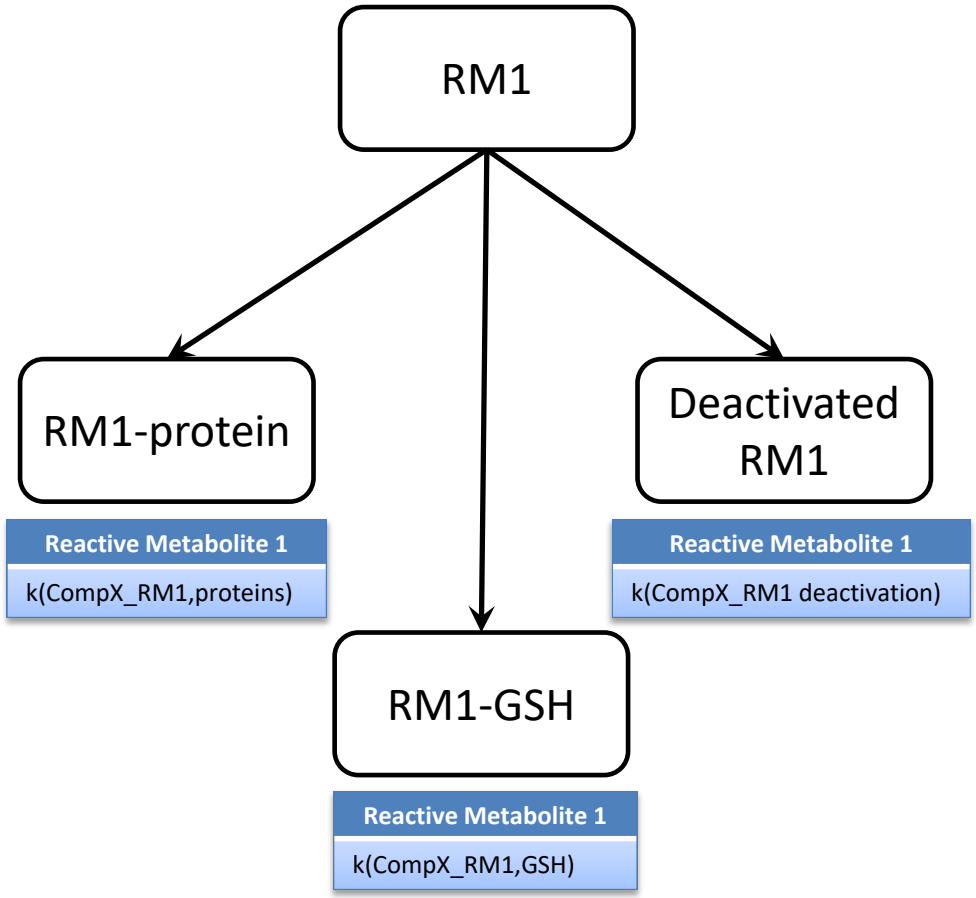
DILIsym Input Panel Document Provides Insight into Data Needed for RM Determination



Inputs or Data Needed	Commonly Used Systems/Assays	DILIsym® Preferred Systems
Concurrent GSH depletion, parent compound disappearance (and metabolite appearance if possible), protein adduct formation,	Primary hepatocytes; immortalized cell lines transfected with cytochrome P450s or other metabolising enzymes; ^{14}C or ^{3}H label for protein adducts	Primary hepatocytes; parent compound should be measured in the cells if possible, but can be estimated if not; comparisons to immortalized cells lines (HepG2) are useful from a metabolite perspective; data from cell lines transfected with drug metabolising enzymes are useful when available
ATP depletion	Primary hepatocytes; immortalized cell lines transfected with cytochrome P450s or other metabolising enzymes; whole-cell ATP	Primary hepatocytes; comparisons to immortalized cells lines (HepG2) are useful from a metabolite perspective; data from cell lines transfected with drug metabolising enzymes are useful when available
ROS/RNS indicators	Primary hepatocytes; immortalized cell lines transfected with cytochrome P450s or other metabolising enzymes; TBARS; peroxynitrite; lipid hydroperoxide, fluorescent probes (DCFDA, DHR123);	Primary hepatocytes; comparisons to immortalized cells lines (HepG2) are useful from a metabolite perspective; data from cell lines transfected with drug metabolising enzymes are useful when available

Reactive Metabolites Can Be Cleared in Several Ways within DILIsym



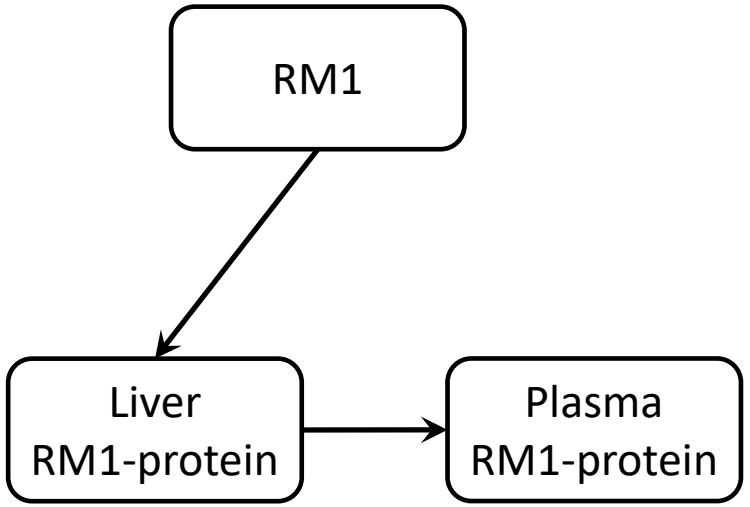
- Reactive metabolites have three potential modes of clearance
  - Adduction to cellular proteins
  - Conjugation with glutathione
  - Deactivation
  - Each is represented by a first-order process relative to the RM
- *In vitro* data determine which method(s) of clearance to model
  - GSH trapping assay can determine whether GSH conjugation is occurring
  - Protein binding is generally present with reactive metabolites
  - Deactivation occurs when the reactive metabolite is an intermediate; downstream metabolites will appear in a microsomal study

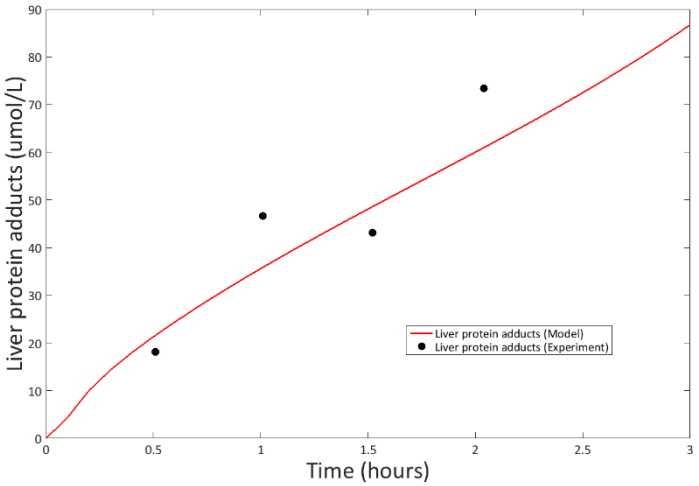
Reactive Metabolite Clearance Assumptions

- Reactive metabolites exist in the liver only
  - RM scaffold cannot be used to represent stable metabolites that require plasma tracking
  - Upon cell death, intracellular RM turns into plasma RM protein adducts
- Deactivated RM and RM-GSH are not cleared from the liver
  - Cumulative amount of each produced on a per-cell basis is calculated over the course of the simulation
  - Not released into the blood stream after cell death

Protein Adducts in DILIsym

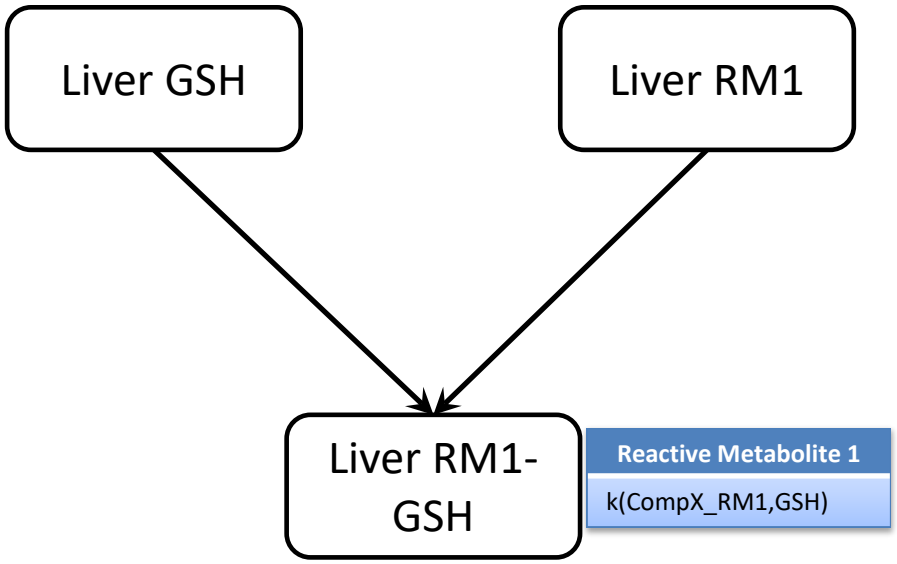
- Protein binding is a first-order process
- Protein adducts can cause toxicity
  - All DILIsym toxicity mechanisms available to protein adducts
  - Adducts do not cause ROS in current APAP model
- Protein adducts are actively transported out of the hepatocyte
  - Adducts cleared from plasma by half-life
- Adducts can serve as a biomarker for validation
  - Example: APAP adducts in rat liver validated with data from Speeg 1985





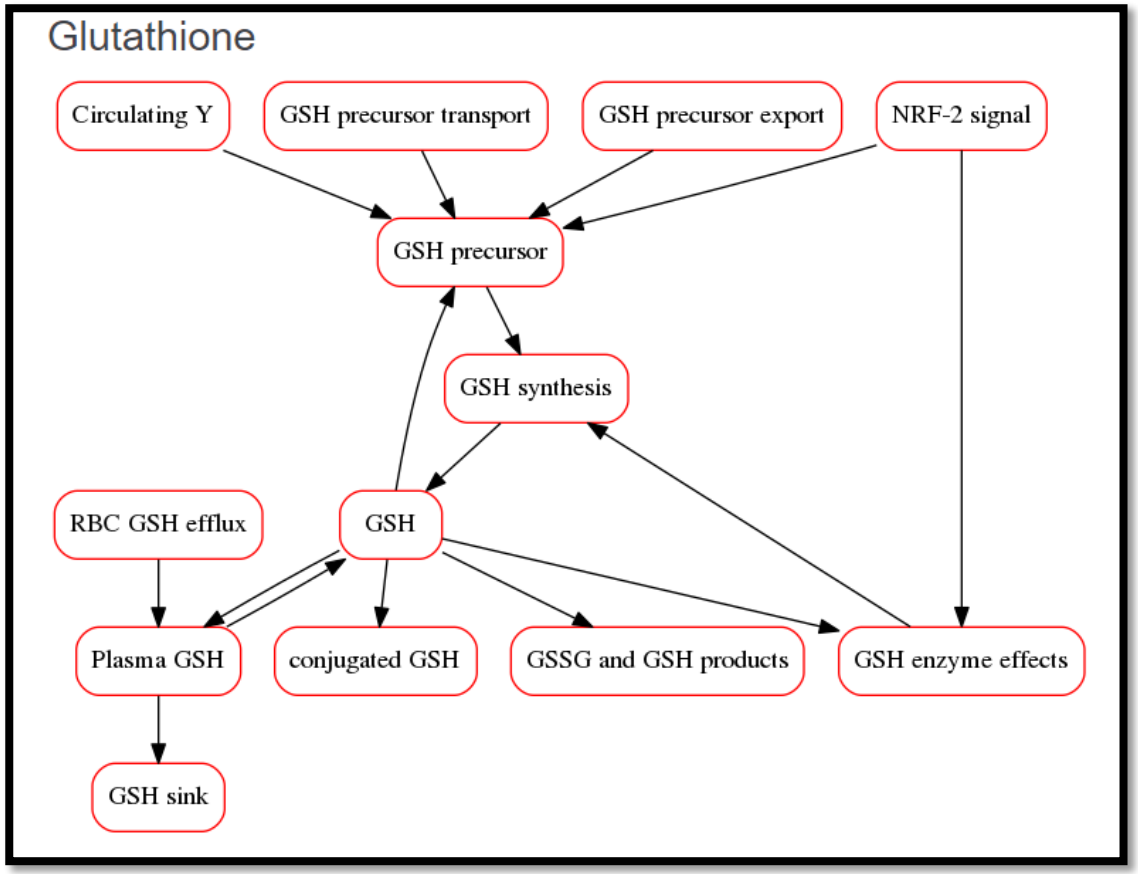
GSH Binds and Neutralizes Reactive Metabolites

- RM-GSH reaction is second-order (first order with respect to both molecules)
  - Allows GSH depletion to occur
- RM-GSH conjugation product is **not** available to cause toxicity within the software
- Rate constant is extremely high in APAP case in order to reflect immediate reaction
  - Other RMs might not conjugate with GSH as readily
  - Rate constant can be determined by using *in vitro* GSH binding assays if expected to be in competition with other RM reactions *in vivo*



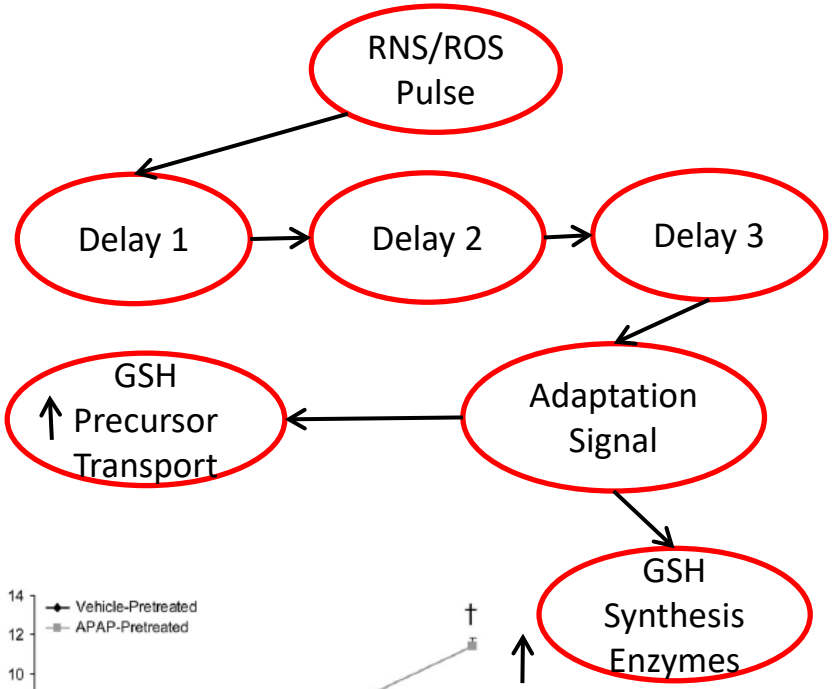
Glutathione (GSH) Sub-model in DILIsym

- DILIsym contains a model of GSH homeostasis and conjugation to reactive metabolites
- ***In DILIsym currently, GSH exists only for conjugation by reactive metabolites***
  - ***GSH is not affected by cellular oxidative stress from other sources***
- Baseline GSH can vary across a population
  - Important for susceptibility to RM-mediated toxicity
- N-acetylcysteine can serve as a GSH precursor, boosting GSH levels
  - Uses existing Compound Y scaffold



ROS Increase Regulates Glutathione Synthesis and Transport Via NRF-2 in DILIsym

- Glutathione synthesis is regulated by the nuclear transcription factor NRF-2
  - NRF-2 increases glutathione precursor transport and glutathione synthesis in reaction to oxidative stress
- NRF-2 activity is delayed after ROS insult
  - Effect lasts for about four days
- NRF-2 activity explains clinically/preclinically observed autoprotection from APAP toxicity
  - Non-lethal doses of APAP protect against following doses of APAP that would otherwise be lethal



Aleksunes Tox Sci 2008

Preclinical Data

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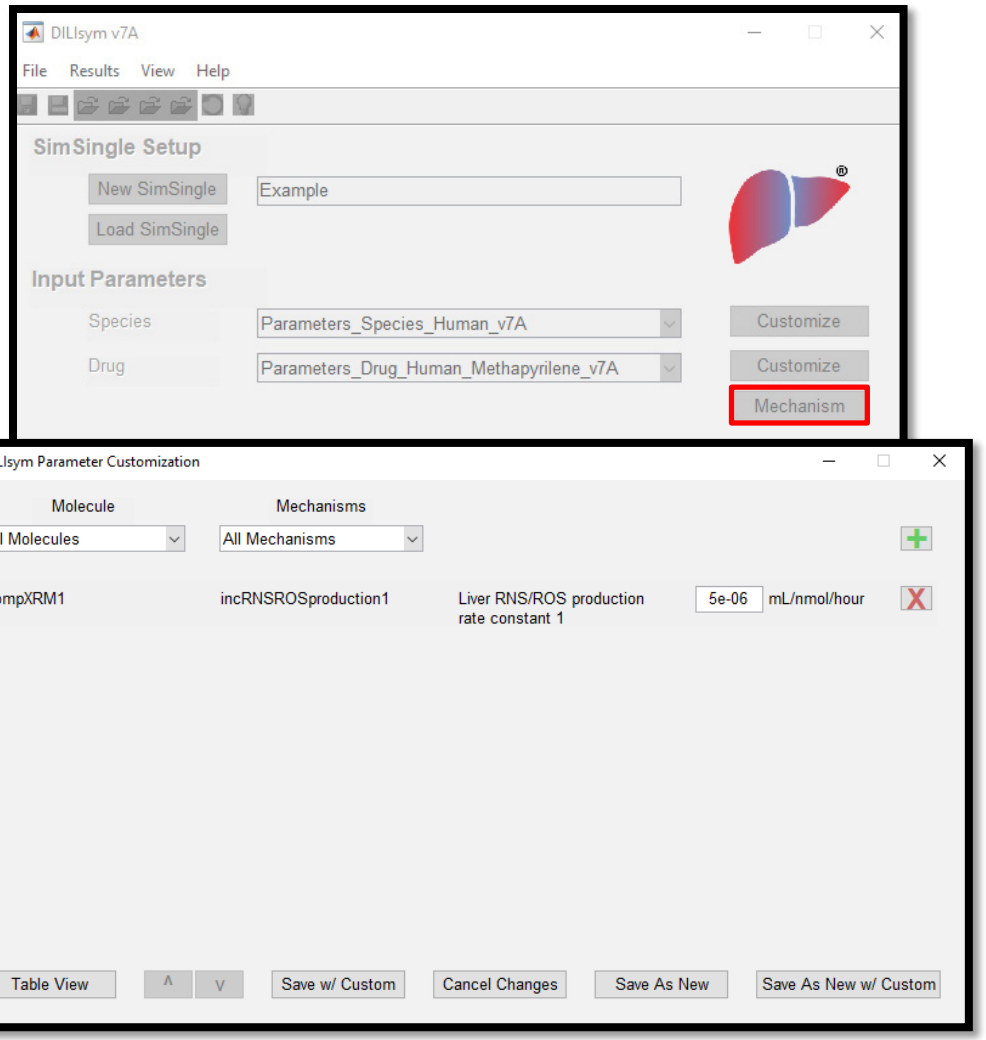
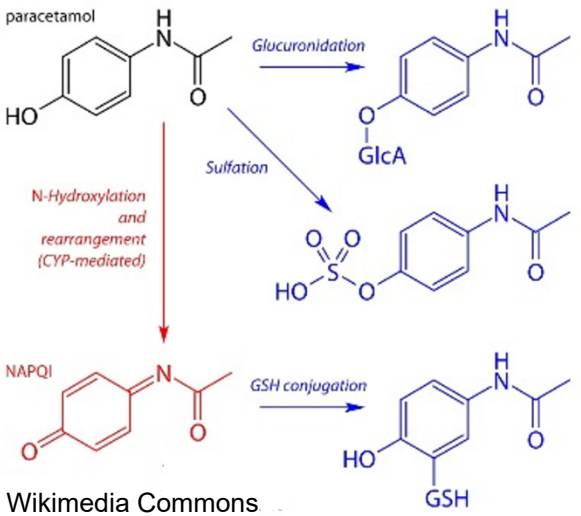
How Reactive Metabolites Can Cause Toxicity in DILIsym

- Reactive metabolites can cause toxicity either alone or as protein adducts
  - **GSH conjugates and deactivated metabolites cannot cause toxicity**
- **Reactive metabolites and protein adducts can be selected as instigators of *any* mechanism currently represented in DILIsym**
- Current assumption for methapyrilene (and APAP, CCl_4) is that reactive metabolites themselves cause oxidative stress
  - Governed by the parameters “Liver RNS/ROS production rate constants”
  - Molecules can be parameterized to produce oxidative stress with a linear or non-linear equation



Acetaminophen Toxicity is Represented Extensively in DILIsym

- Acetaminophen (APAP) is the drug most commonly implemented in DILI in the US and Europe
- APAP is metabolized into three metabolites, including NAPQI, which is reactive
  - Significant species differences in metabolism
- GSH quenches NAPQI easily; toxicity occurs when GSH is depleted and NAPQI can accumulate, causing oxidative stress
- Oxidative stress model in DILIsym was calibrated using APAP data in rats, mice, and humans
  - Immune response model also frequently leverages data from the APAP literature



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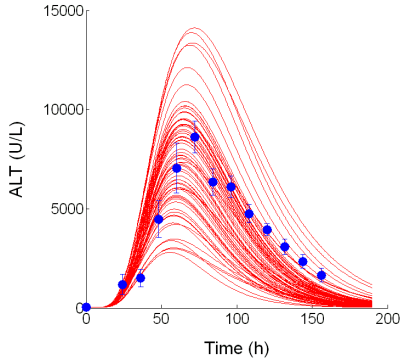

Simulations Results and Clinical Data

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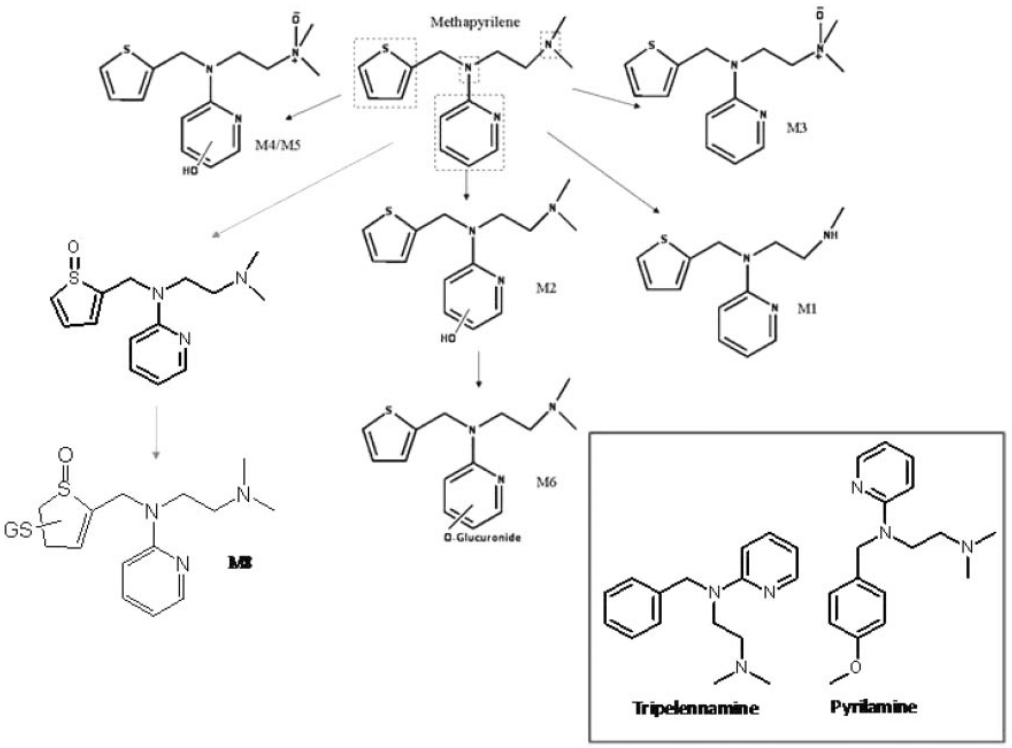
Representing Methapyrilene Requires Combining Several Metabolites from Microsome Studies

- Graham 2008 contains microsome metabolism data used to parameterize methapyrilene metabolism parameters
  - Percentage of initial incubation of MP transformed into various metabolites was reported
- V_{max} values are determined by calculating the values of V_{max} that produce the appropriate percentages after the correct amount of time
  - K_m values are assumed from reported values for similar metabolism pathways
  - K_m could be calculated using microsome incubations at different concentrations; in practice, these data rarely exist
- GSH depletion dynamics similar to acetaminophen
  - Can assume same GSH binding rate constant



Graham JPET 2008

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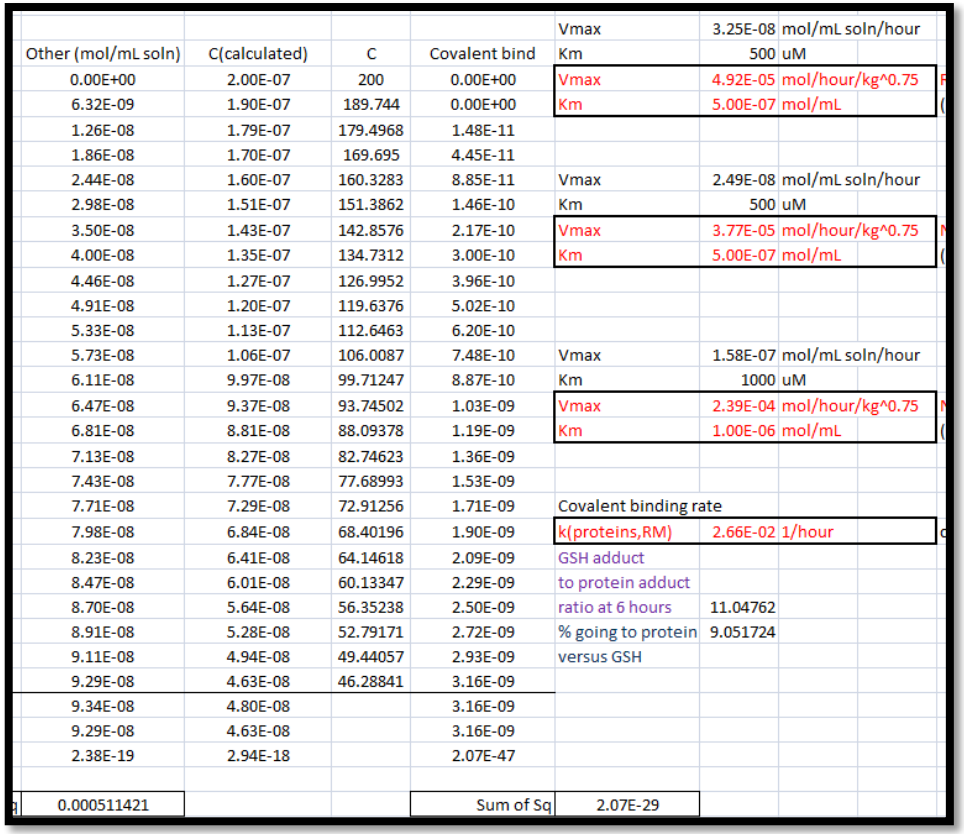
Drug Metabolism Flux Data is Optimal for Determination of Reactive Metabolite Generate Rates

- Microsomal metabolism data is key to the determination of reactive metabolite generation rates
  - RM and its products are often not easily measured *in vivo*
  - Also key to determining GSH binding rate, protein adduction rate, etc.
  - Training sessions contain detailed steps for converting microsomal metabolism data to V_{max} and K_m for input into DILIsym
- *In vivo* data can be used to eliminate certain pathways
  - Furosemide example: no GSH depletion *in vivo* means that pathway can be ignored
- Mechanistic data are still required for determining active toxicity pathways
  - ROS is usually the appropriate pathway but not always



				Vmax	3.25E-08	mol/mL soln/hour
Other (mol/mL soln)	C(calculated)	C	Covalent bind	Km	500	uM
0.00E+00	2.00E-07	200	0.00E+00	Vmax	4.92E-05	mol/hour/kg^0.75
6.32E-09	1.90E-07	189.744	0.00E+00	Km	5.00E-07	mol/mL
1.26E-08	1.79E-07	179.4968	1.48E-11			
1.86E-08	1.70E-07	169.695	4.45E-11			
2.44E-08	1.60E-07	160.3283	8.85E-11	Vmax	2.49E-08	mol/mL soln/hour
2.98E-08	1.51E-07	151.3862	1.46E-10	Km	500	uM
3.50E-08	1.43E-07	142.8576	2.17E-10	Vmax	3.77E-05	mol/hour/kg^0.75
4.00E-08	1.35E-07	134.7312	3.00E-10	Km	5.00E-07	mol/mL
4.46E-08	1.27E-07	126.9952	3.96E-10			
4.91E-08	1.20E-07	119.6376	5.02E-10			
5.33E-08	1.13E-07	112.6463	6.20E-10			
5.73E-08	1.06E-07	106.0087	7.48E-10	Vmax	1.58E-07	mol/mL soln/hour
6.11E-08	9.97E-08	99.71247	8.87E-10	Km	1000	uM
6.47E-08	9.37E-08	93.74502	1.03E-09	Vmax	2.39E-04	mol/hour/kg^0.75
6.81E-08	8.81E-08	88.09378	1.19E-09	Km	1.00E-06	mol/mL
7.13E-08	8.27E-08	82.74623	1.36E-09			
7.43E-08	7.77E-08	77.68993	1.53E-09			
7.71E-08	7.29E-08	72.91256	1.71E-09	Covalent binding rate		
7.98E-08	6.84E-08	68.40196	1.90E-09	k(proteins,RM)	2.66E-02	1/hour
8.23E-08	6.41E-08	64.14618	2.09E-09	GSH adduct		
8.47E-08	6.01E-08	60.13347	2.29E-09	to protein adduct		
8.70E-08	5.64E-08	56.35238	2.50E-09	ratio at 6 hours	11.04762	
8.91E-08	5.28E-08	52.79171	2.72E-09	% going to protein	9.051724	
9.11E-08	4.94E-08	49.44057	2.93E-09	versus GSH		
9.29E-08	4.63E-08	46.28841	3.16E-09			
9.34E-08	4.80E-08		3.16E-09			
9.29E-08	4.63E-08		3.16E-09			
2.38E-19	2.94E-18		2.07E-47			
0.000511421			Sum of Sq	2.07E-29		

Overview of Oxidative Stress in DILIsym

- Hepatocytes are generating reactive oxygen and nitrogen species (RNS/ROS) and clearing them constantly
  - Antioxidants in liver take care of baseline oxidative stress, facilitating homeostasis
- Both reactive metabolites and stable molecules (parent or metabolite) can increase concentrations of RNS/ROS in the liver
  - Can be caused by molecules interfering with mitochondrial function or disturbing the antioxidant pool, for example
  - This disturbs the homeostasis, causing oxidative stress
- Build-up of saturated fatty acids leads to oxidative stress
- Oxidative stress can lead to apoptosis or necrosis
  - Dependent on cellular energy state

Limitations of Oxidative Stress Model

- Current oxidative stress sub-model represents antioxidant status as a first-order clearance term of RNS/ROS
  - Inter-individual variability included in SimPops
  - Explicit antioxidant activity, effect of diet, etc. not included currently
- GSH sub-model only impacts reactive metabolites via buffering (conjugation)
  - Cellular glutathione status does not act on RNS/ROS directly
  - GSH depletion cannot be caused by parent drug or non-reactive metabolites
- RNS/ROS production is independent of mitochondrial dysfunction and bile acid accumulation
  - Physiologically, mitochondrial dysfunction can lead to RNS/ROS; this is not modeled within DILIsym currently
  - For novel compounds, we take this into account by measuring both RNS/ROS production and mitochondrial dysfunction as part of the Input Panel