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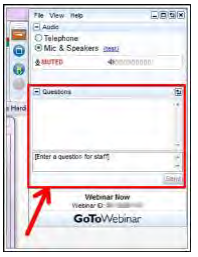

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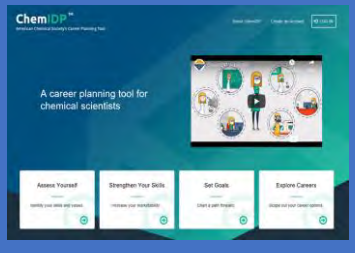

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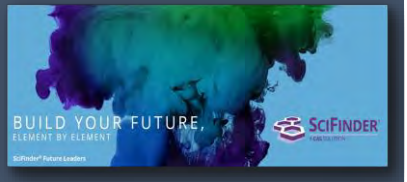

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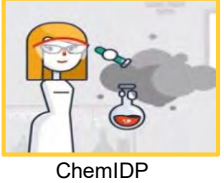
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What You Will Learn

- Not all caramels are the same and why
- The difference between gummy and jelly candies
- What candy corn is actually made of

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Moderator: Diane Grob Schmidt, 2015 ACS President

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- The many sources of funding and their impact on ownership
- The importance of milestone achievements for valuation purposes
- The criteria and terms that investors use to make investing decisions

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Tuesday, November 10, 2020 at 2-3pm ET
Speaker: Fatima Dainkeh, She+ Geeks Out
Moderator: Paula Christopher, American Chemical Society

Register for Free!

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- What a microaggression is, looks like, and how it manifests
- How to respond to microaggressions if you experienced one or how to respond to someone who has shared that they have been offended by something you did or said
- How to respond to a microaggression if you witnessed one, but were not an active participant

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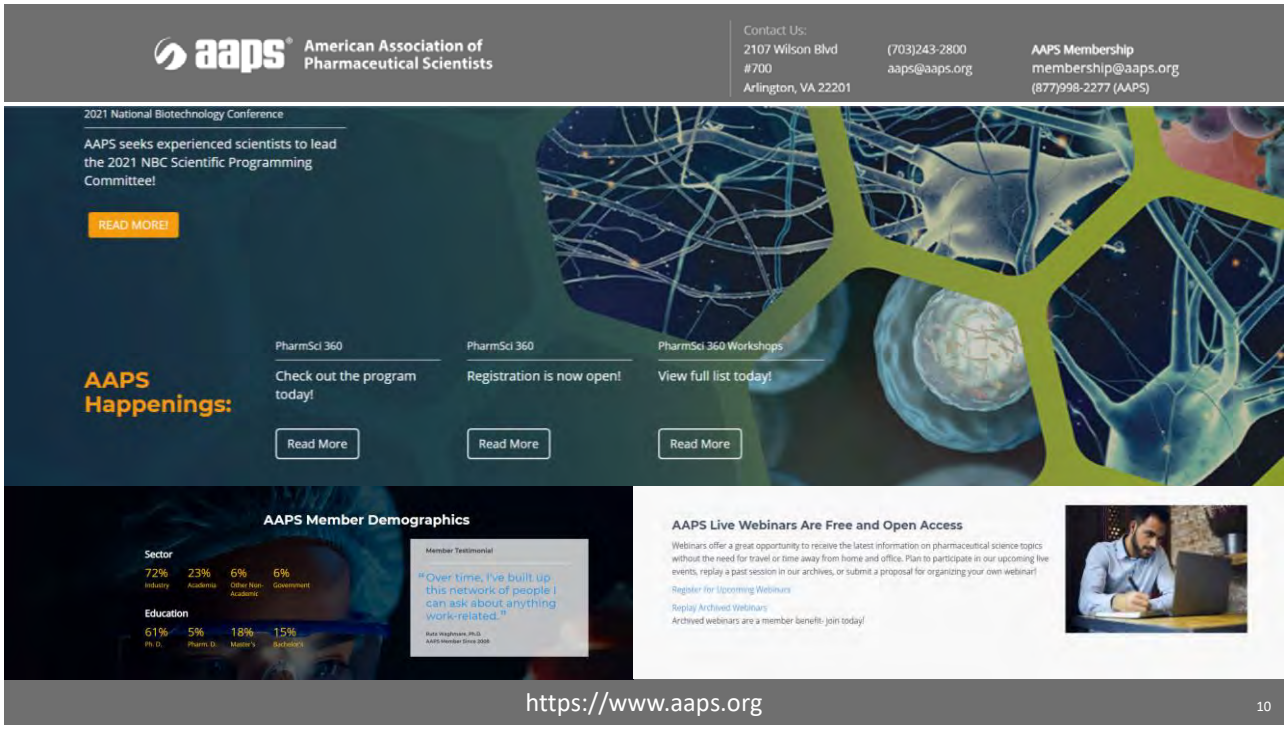

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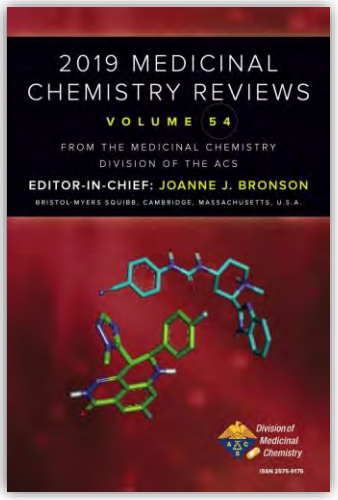

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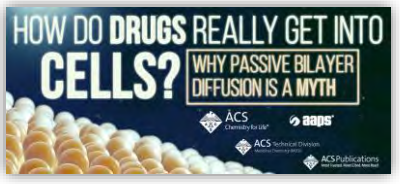
https://www.acs.org/content/acs/en/acs-webinars/drug-discovery/passive-permeability.html



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Mitigating Drug-Induced Liver Injury: Assessing Mitochondrial Toxicity and Reactive Metabolism

Kevin Coe
Senior Principal Scientist, Janssen Pharmaceutical Companies of Johnson & Johnson

Yvonne Will
Vice President, Predictive and Investigative Toxicology (Nonclinical Safety), The Janssen Pharmaceutical Companies of Johnson & Johnson

Kaushik Mitra
Director, Department of Drug Metabolism and Pharmacokinetics; DMPK Therapeutic Area Lead, Cardiovascular and Metabolic Diseases; Head, Biotransformation Sciences, Janssen Research & Development

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Reactive Metabolism Trapping Applied to Drug Discovery: Applications & Present Challenges

Kevin J. Coe
Janssen Research & Development, Discovery Sciences, DMPK
29 October 2020

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Outline

- **Introduction**
  - Drug Induced Liver Injury (DILI)
  - Role of Reactive Metabolism in Drug Safety
- **Factors Influencing Adduct Detection**
  - Instrumentation
  - Software
  - Test Systems
- **Case Studies to Address Reactivity**
- **Present Challenges**
- **Conclusions**

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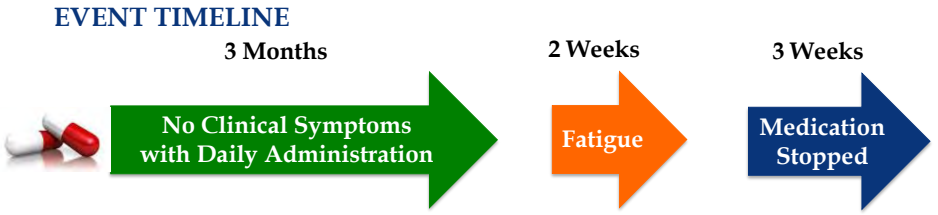
Idiosyncratic Hepatotoxicity: Severe & Unpredictable

- Patient is administered a new anti-diabetic agent with a novel mechanism of action to treat her Type II diabetes



Murphy EJ, et al., Dig Dis Sci, 2000

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Idiosyncratic Hepatotoxicity: Severe & Unpredictable

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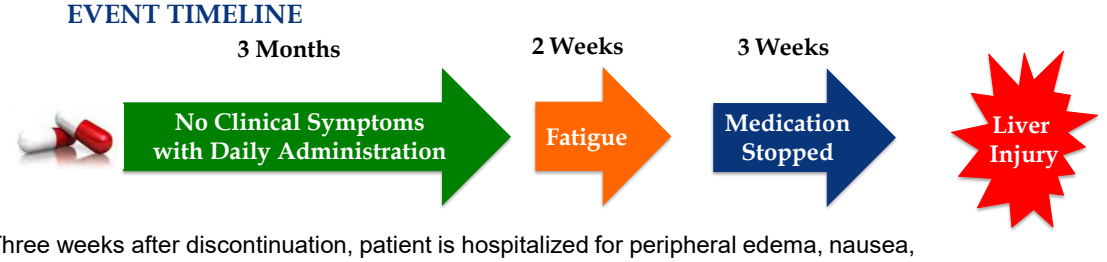

- Three weeks after discontinuation, patient is hospitalized for peripheral edema, nausea, emesis, and metabolic acidosis
- A liver biopsy reveals hepatic necrosis requiring a liver transplant

Murphy EJ, et al., Dig Dis Sci, 2000

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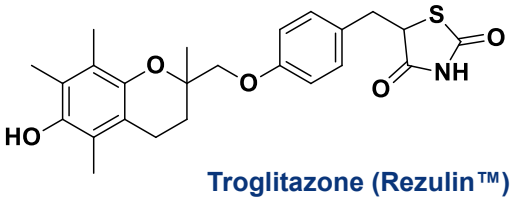
Troglitazone: A Case Study of an Idiosyncratic Hepatotoxicant



Troglitazone (Rezulin™)
600 mg QD

- Troglitazone was a first in class drug to designed to activate peroxisome proliferator-activated receptors (PPARs) to treat Type II diabetes
- Pre-marketing, ~2,500 patients were dosed with only mild increases in ALT observed in 1.9 % of patients and only overt liver injury in two patients
- Post-marketing, ~ 2 million patients were administered troglitazone and within three years of use 63 patients died from hepatotoxicity associated with drug
- In March 2000, three years after its launch, troglitazone was withdrawn

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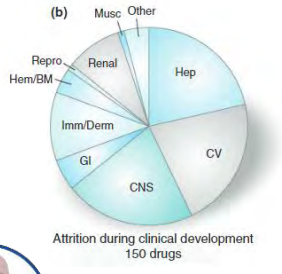
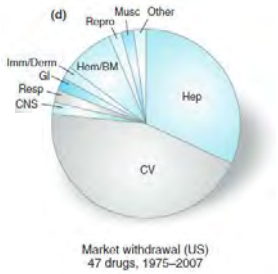
Drug Induced Liver Injury (DILI)

- The liver is often an organ susceptible to drug induced liver injury (DILI) given its role to process and eliminate drugs
- DILI is responsible for > 50% of acute liver failure cases
- Excluding acetaminophen, DILI can be attributed to 14% of cases which can bear up 10% mortality rate
- DILI is a major reason for drug attrition in early pre-clinical safety assessment, during clinical trials, and post-marketing
- Between 1975 – 2007, DILI accounts for 32% of drug withdrawals



Attrition during clinical development
150 drugs



Market withdrawal (US)
47 drugs, 1975–2007

Drug Disc. Today V19, **2014**, 1131

Recent late-stage failures in clinical trials due to DILI

- ✓ Two Merck migraine drugs, MK-3207 (Ph2b) and MK-0974 (Ph3), discontinued between 2009-2011
- ✓ GPR40 agonists TAK-875 (Takeda) & MK-8666 (MSD) discontinued in Ph3/Ph1
- ✓ J&J BACE inhibitor, atabecestat, terminated in Ph2b/Ph3 in 2018

(Stevens and Baker, *Drug Discov Today*, 2009)

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Why is Predicting DILI Challenging?

- DILI is often idiosyncratic
  - Extremely rare occurrence (< 1 out of 10,000 patients)
  - May require long latency periods to manifest (weeks to months)
  - Not anticipated from drug's mechanism of action
- No apparent dose relationship
- No common risk factor that can be broadly applied to proactively identify those patients most susceptible
- Preclinical species cannot reliably reproduce clinical DILI
- Limited mechanistic understanding for cause compounded by involvement of multifactorial processes and complex mechanisms

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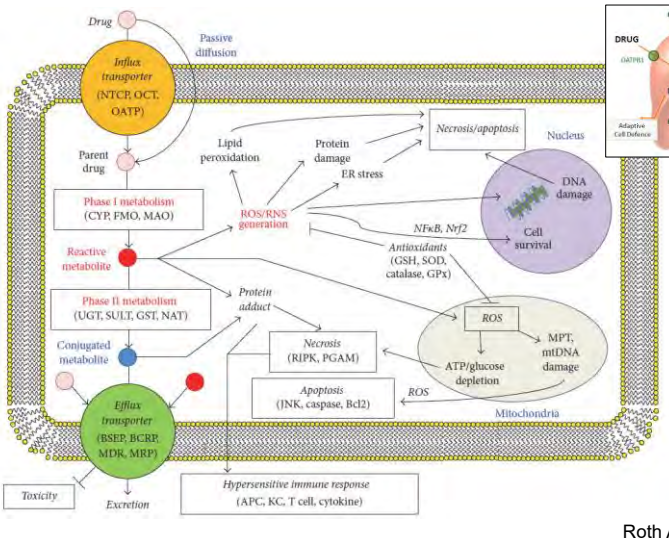
Drug Induced Liver Injury (DILI) – One Organ, Multiple Mechanisms



Roth A, *BioMed Research Int*, 2017

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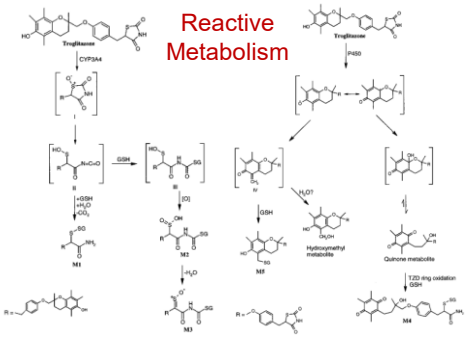
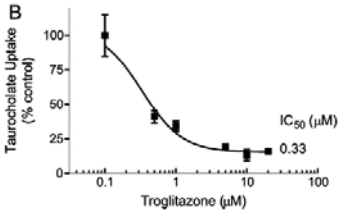
One Drug, Multiple Mechanisms: Troglitazone

Reactive Metabolism



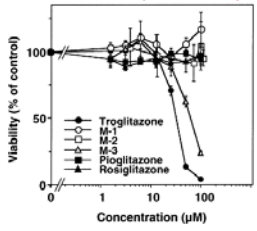
Kassahun K et al, *Chem Res Toxicol*, 2001

Transporter Inhibition



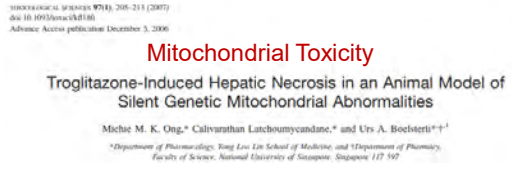
Marion TL et al, *Mol Pharmaceutics*, 2007

Cellular Cytotoxicity



Yamamoto Y et al, *Life Sciences*, 2001

Mitochondrial Toxicity



Troglitazone-Induced Hepatic Necrosis in an Animal Model of Silent Genetic Mitochondrial Abnormalities

Michie M. K. Ong,* Calivarathan Latchoumycandane,* and Urs A. Boelsterli*,†,1

*Department of Pharmacology, Yong Loo Lin School of Medicine, and †Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore 117 597

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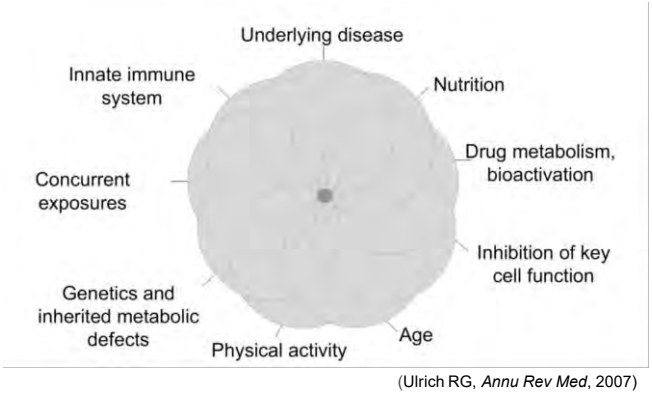
One Patient, Multiple Determinants



(Ulrich RG, *Annu Rev Med*, 2007)

- Multifactorial requirements, often patient-specific, are likely required for onset, posing an obstacle to predict prospectively patients at risk
- As consequence, drug-related risk factors remain the focus preclinically to minimize DILI potential

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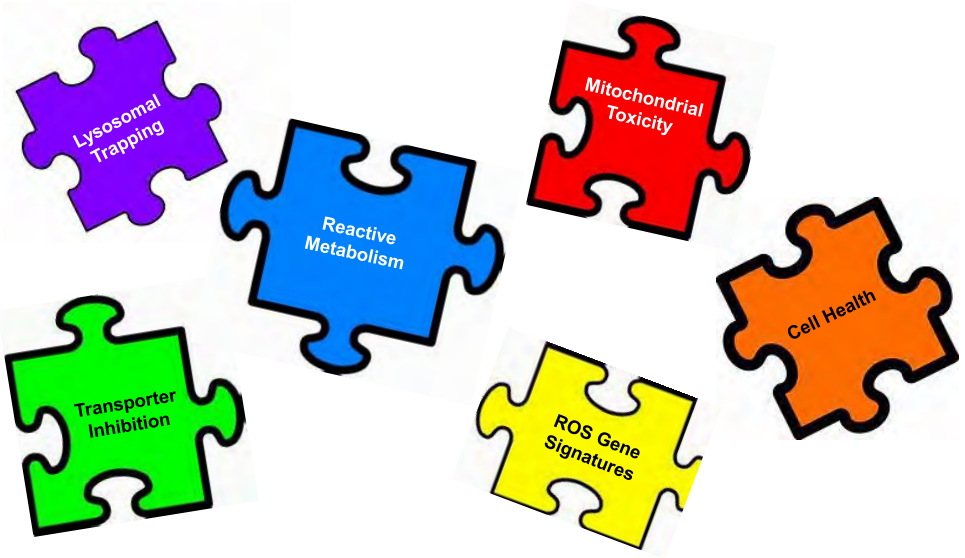
Multiple Mechanisms, Multiple Assays – How Do We Integrate?



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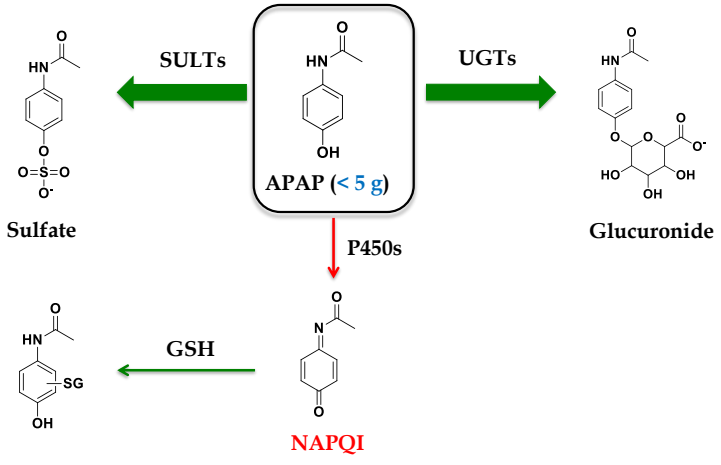
Drug Metabolism – A Benign Process to Eliminate Drugs



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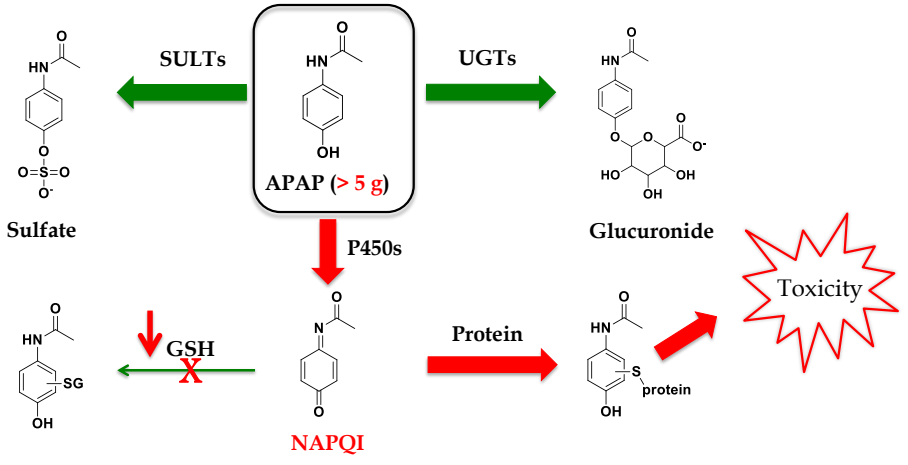
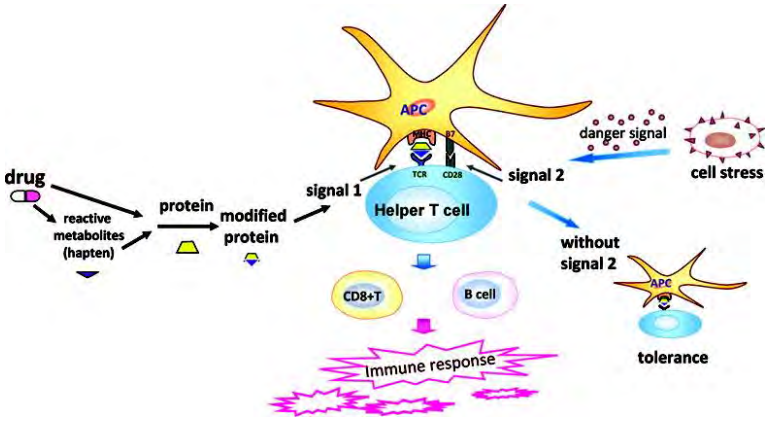
Drug Metabolism – Bioactivation to Cause DILI



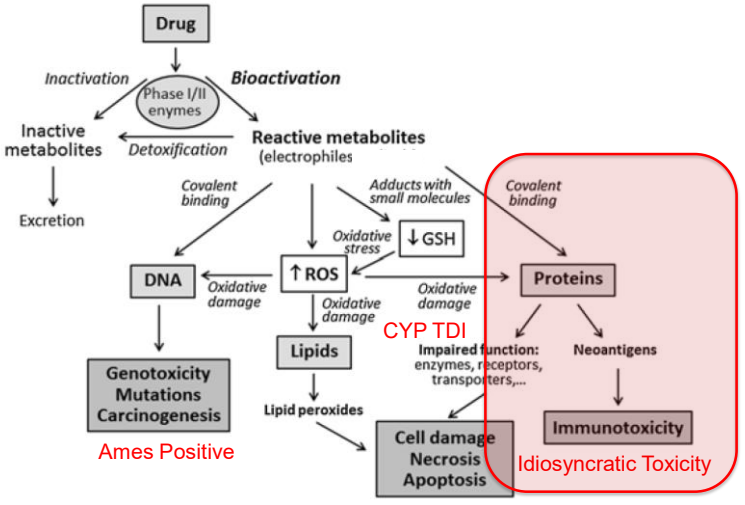
Haptenization Believed to be Critical Determinant for IDRs



- Autoantibodies can be formed to Cytochrome P450s involved in drug bioactivation

Uetrecht J; *Chem. Res. Toxicol.* **2008,** 21, 84-92.

Reactive Metabolism Responsible for Multiple Mechanisms of Toxicity



Gómez-Lechón, *J Appl Toxicol*, 2016

Toleration Studies / Cytotoxicity

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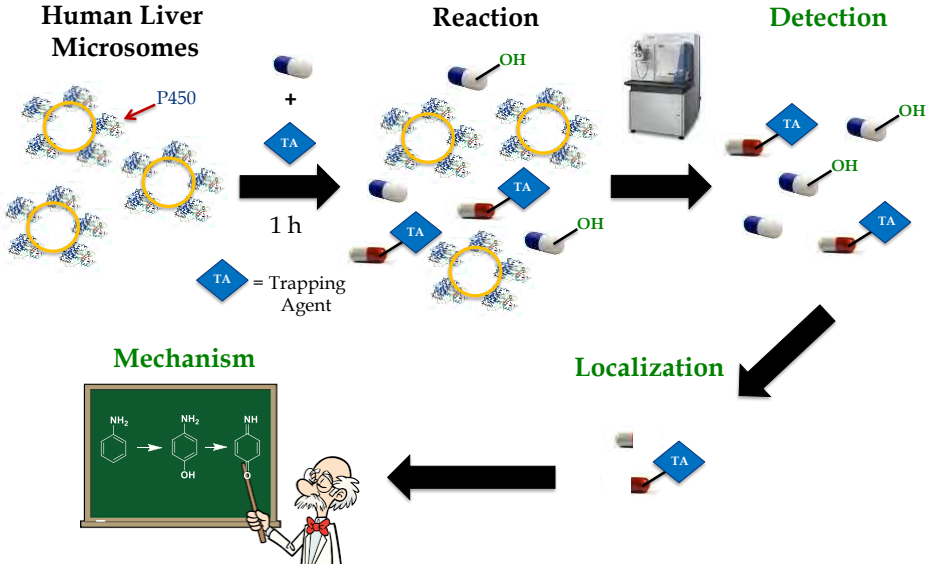
Address Reactive Metabolism in Trapping Assays



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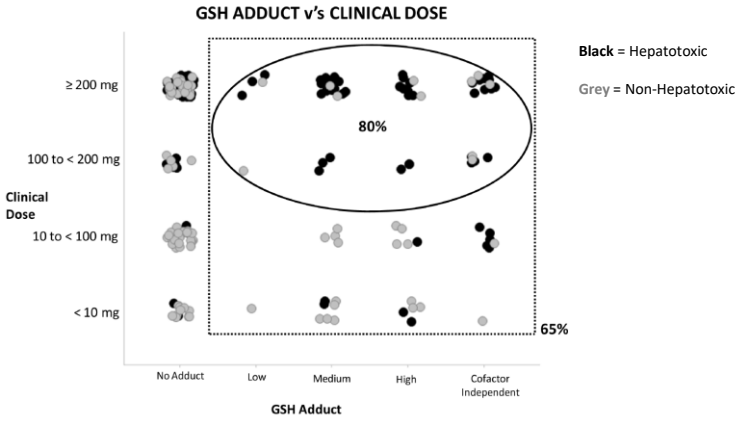
Trapping Assays Can Improve DILI Prediction



- Best predictor of DILI potential is dose (> 100 mg dose has greater likelihood for DILI)
- Increased likelihood for DILI if high dose drug forms GSH adducts

(Sakatis MZ, *Chem Res Toxicol*, 2012)

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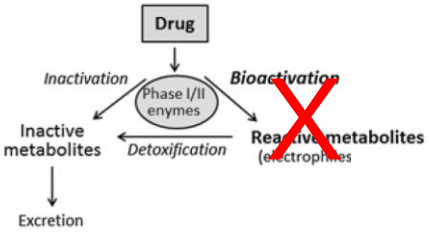
Consequences of Reactive Metabolism Minimized by Preventing Their Formation



Gómez-Lechón, *J Appl Toxicol*, 2016

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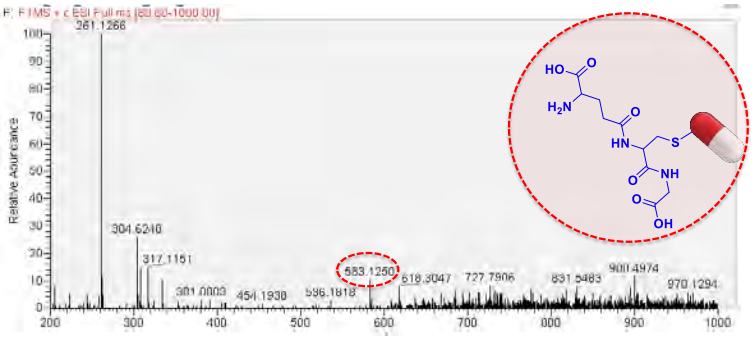
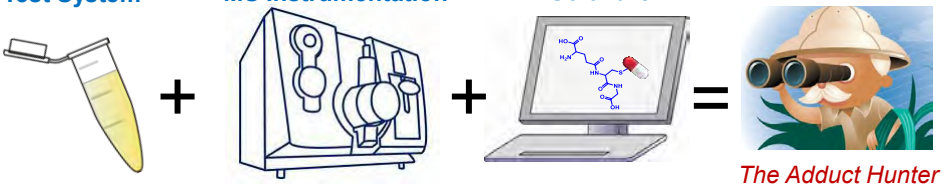
Factors Influencing Adduct Detection



Test System + **MS Instrumentation** + **Software** = *The Adduct Hunter*



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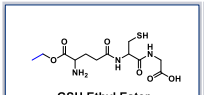
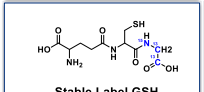
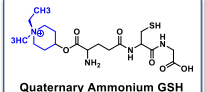
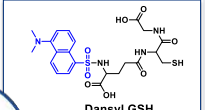
Modified GSH Trapping Agents to Aide Detection

GSH Analog	Utility	Reference(s)
 GSH Ethyl Ester	• Increased MS sensitivity (~ 10-fold > GSH) • Greater hydrophobicity of adducts increases retention for SPE clean-up and LC • Reduces endogenous background by monitoring *m/z* 300 in (-) Mode	• Soglia JR et al. *J Pharm Biomed Anal.* 2004 • Wen B & Fitch WL. *J Mass Spectrom* 2009
 Stable Label GSH	• Isotopic pattern creates unique signature for adducts to minimize false positive and allow for structure-independent mining • Enables MS techniques for adduct identification and data-rich spectra for structural elucidation	• Yan Z & Caldwell G. *Anal Chem* 2004 • Mutlib A et al. *Rapid Commun Mass Spectrom* 2005
 Quaternary Ammonium GSH	• Fixed positive charge permits for semi-quantitation of GSH adduct abundance when paired with internal standards	• Soglia JR et al. *Chem Res Toxicol.* 2006
 Dansyl GSH	• Dansyl group permits for fluorescent detection for quantification to appreciate magnitude of reactivity and differentiate structural analogs	• Gan J et al. *Chem Res Toxicol.* 2005

MS Instrumentation

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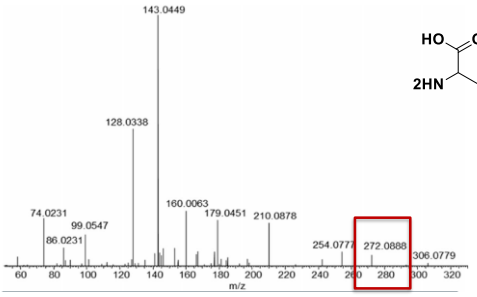
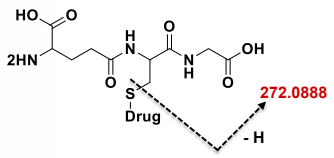
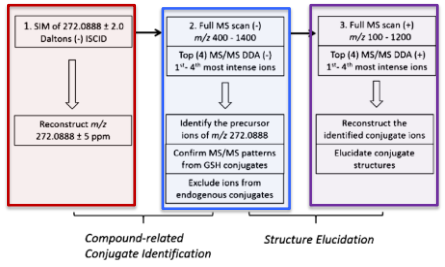
MS Instrumentation Techniques (An illustration)

MS Instrumentation





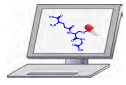
- Select Ion Monitoring for 272 *m/z* using in-source CID (Negative Mode)
- Negative Mode Full Scan + MS2 DDA
- Positive Mode Full Scan + MS2 DDA

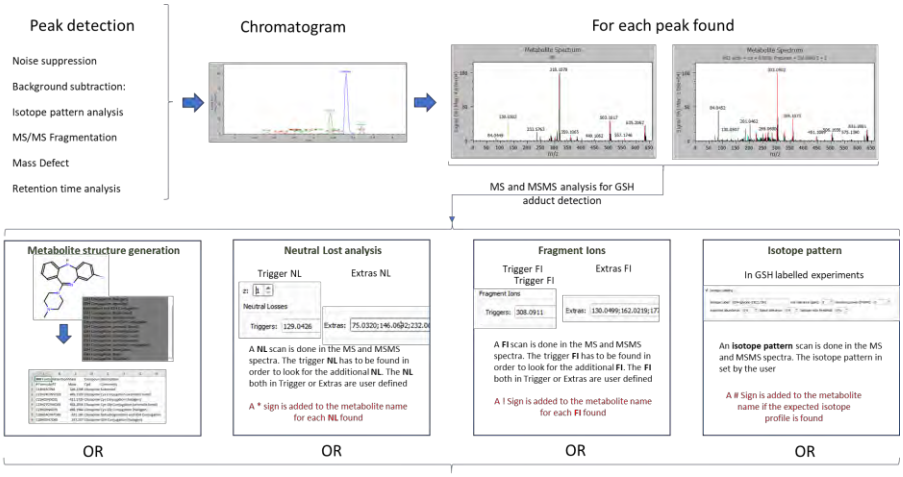


Adaptation from Wang Z *et al*, *Anal Bioanal Chem*, 2018

Software Tools Accelerate Data Mining

Software





Slide courtesy of Ismael Zamora Rico, Molecular Discovery, LTD

Low Turnover Drugs Push Need for Increased Sensitivity

Test System

- **Program where GSH adducts detected despite low metabolic turnover**

Compound	HLM $t_{1/2}$ (min)	GSH Adduct	% Parent Remaining	Metabolites Detected	Greatest Metabolite Level (MS counts - Area)
1	>180	NO	95	Yes	2.20E+07
2	>180	YES	95	Yes	2.60E+07
3	>180	YES	96	Yes	2.70E+07
4	>180	NO	96	Yes	1.40E+07
5	>180	YES	96	Yes	2.40E+07
6	>180	YES	96	Yes	9.90E+07
7	>180	YES	96	Yes	3.70E+07
8	>180	NO	98	No	N/A
9	>180	YES	99	Yes	9.50E+05
10	>180	YES	99	Yes	5.10E+06

- **Program where GSH adducts missed using conventional models due to low turnover**
  - Lead molecule without evidence for GSH adducts in HLMs or suspension hepatocytes
  - Clearance primarily metabolic in rodents with thiol conjugation a major metabolic pathway
  - Confirmation in human long-term coculture models of operative bioactivation pathway

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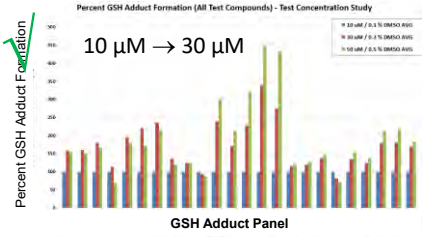
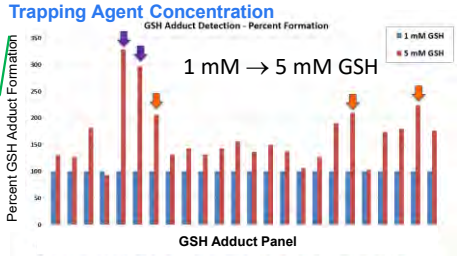
Enhanced HLM Conditions Identified for Detection

Test System

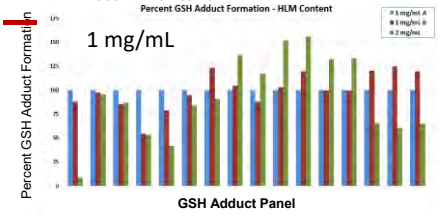
Test Compound Concentration



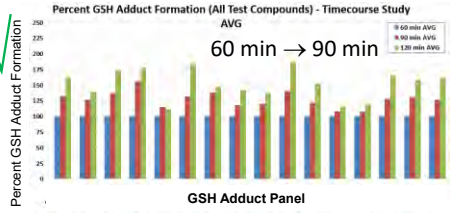
Trapping Agent Concentration



HLM Protein Content



Time



- Optimization studies in HLMs using low turnover JNJ test sets demonstrate modest benefit in adduct detection through fine tuning certain assay parameters

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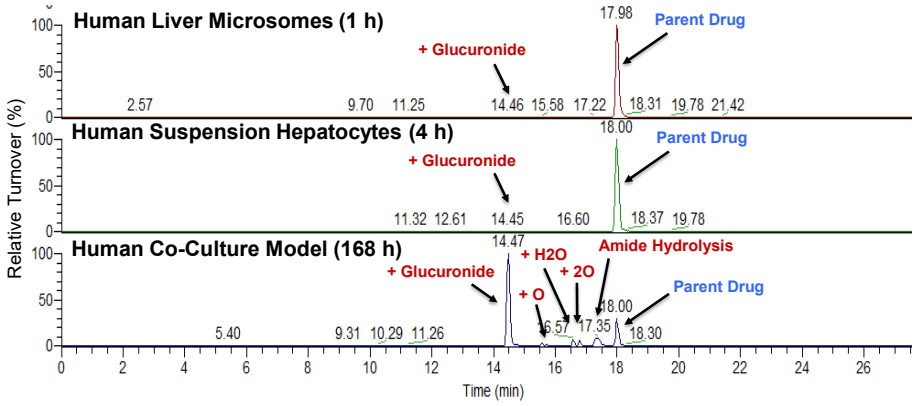
New In Vitro Models Have Improved Metabolic Capability

Test System

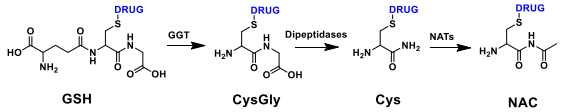


- Long term hepatocyte co-culture models provide increased metabolite formation for very low turnover drugs compared to conventional in vitro metabolic systems

Improvements in Detection Possible

Test System

Test Compound	HLM Half-Life (min)	Adduct	Rat LMs	Human LMs	Enhanced HLMs (HLM*)	Human Co-Culture	System with Adduct Detection
1	> 180	+ GSH - HF + O	√ (20-fold ↑)	√	√	n/d	RLM, HLM, HLM*, Co-Culture
		+ Cysgly - HF + O	n/d	n/d	n/d	√	
2	167	+ GSH + O	√ (25-fold ↑)	n/d	√	n/d	RLM, HLM*, Co-Culture
		+ Cysgly + O	n/d	n/d	n/d	√	
3	> 180	+ GSH - HF + O	√ (8-fold ↑)	n/d	√	n/d	RLM, HLM*, Co-Culture
		+ Cysgly - HF + O	√ (13-fold ↑)	n/d	√	n/d	
		+ Cys - HF + O	n/d	n/d	n/d	√	
4	> 180	+ GSH - HF + O	√ (44-fold ↑)	n/d	√	n/d	RLM, HLM*, Co-Culture
		+ Cysgly - HF + O	√	n/d	n/d	n/d	
		+ Cys - HF + O	n/d	n/d	n/d	√	
5	> 180	+ GSH - 2H	√	n/d	n/d	n/d	RLM, Co-Culture
		+ Cys - 2H	n/d	n/d	n/d	√	
		+ NAC - 2H	n/d	n/d	n/d	√	
6	122	+ GSH + O	√ (100-fold ↑)	NT	√	n/d	RLM, HLM*



The Adduct Hunter

The Adduct Doctor

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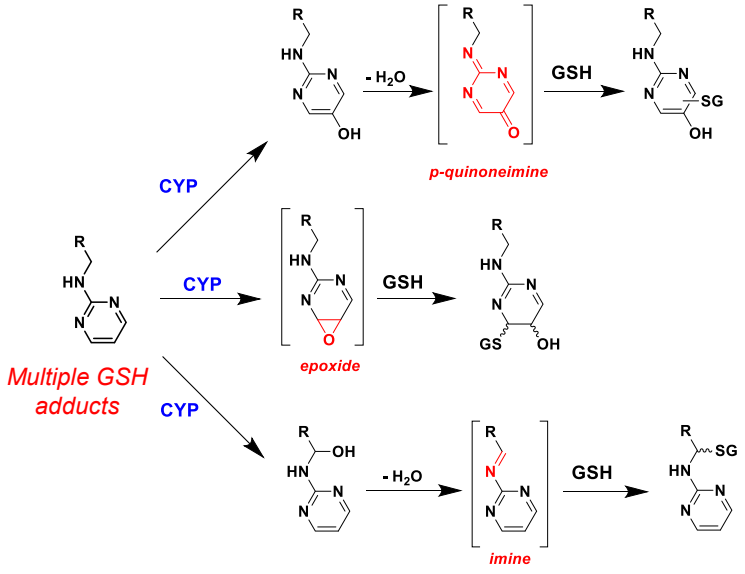
Case Study 1



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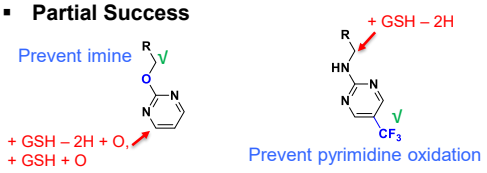
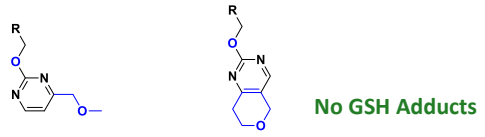
Strategies to Prevent Bioactivation

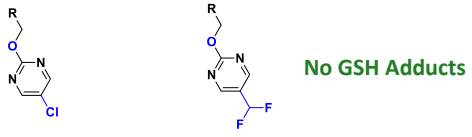
- **Partial Success**



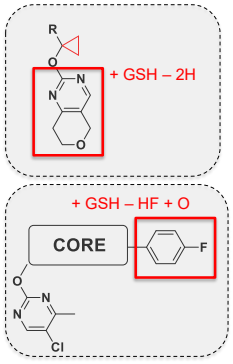
- **Success Strategy 1 – Introduce Alternative Site of Metabolism**



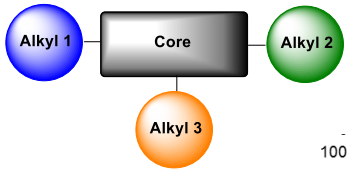
- **Success Strategy 2 – Introduce Metabolic Blocking Groups**

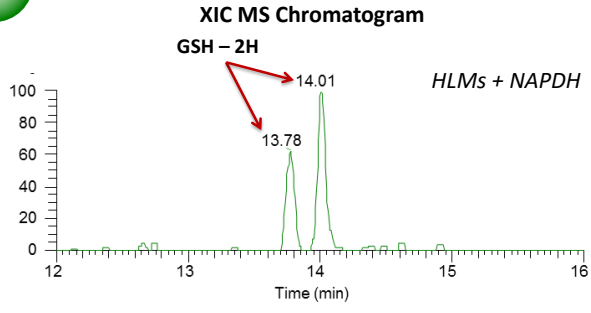


however…



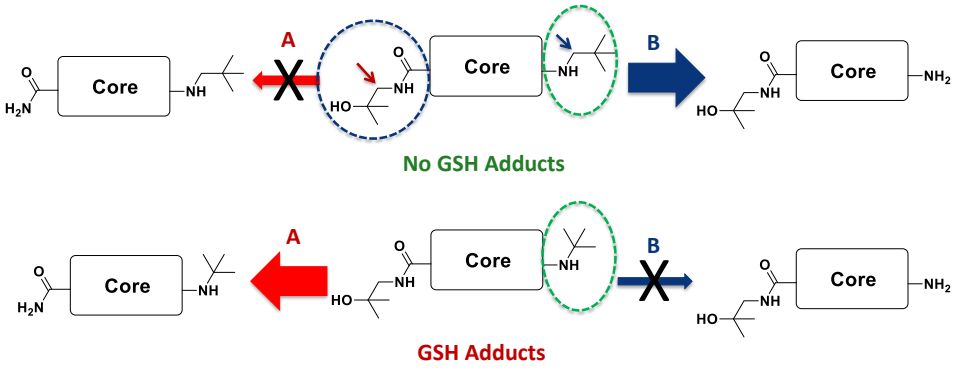
Case Study 2





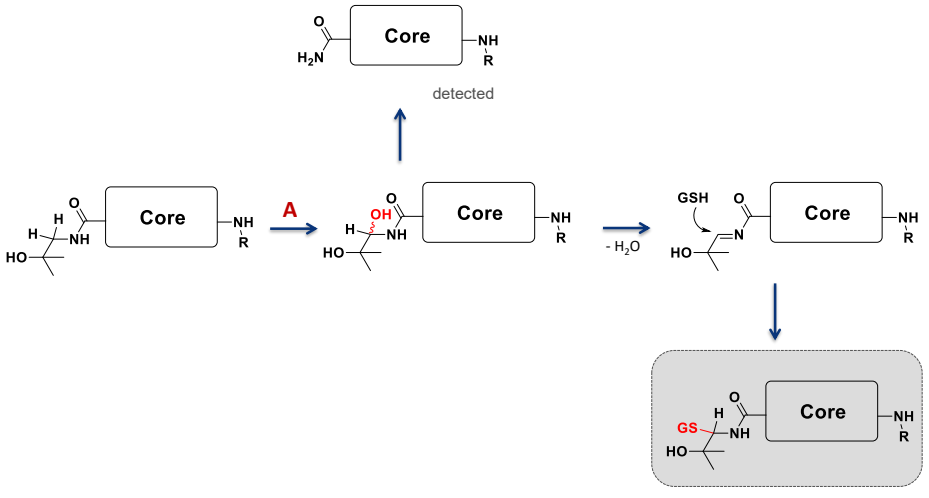
- Lead chemotype forms GSH adducts requiring NADPH suggestive of bioactivation
- MS/MS spectra supports adduct at an aliphatic group but unclear which one
- SAR efforts unsuccessful to implicate the responsible alkyl substructure

N-Dealkylation Pathways Influence Reactivity Potential

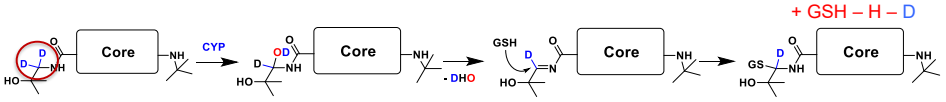


GSH adducts likely to form when *N*-dealkylation pathway A was major

N-Dealkylation Pathway Involved in Bioactivation



Utilize Deuterated Analog to Test Mechanism



Possible Outcomes & Conclusions

1. **No GSH adducts detected**
   → ***supports proposed mechanism***

2. **GSH adducts detected <u>with</u> loss of a deuterium atom**
   → ***supports proposed mechanism***

3. **GSH adducts detected <u>without</u> loss of a deuterium atom**
   → ***refutes proposed mechanism***
   → ***GSH adduct forms elsewhere***

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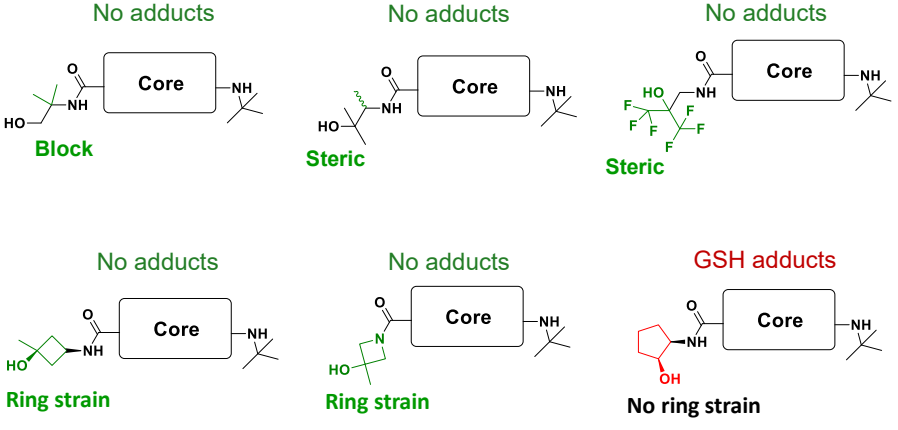
Strategies to Prevent Reactive Metabolism



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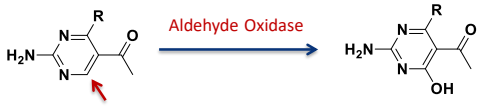
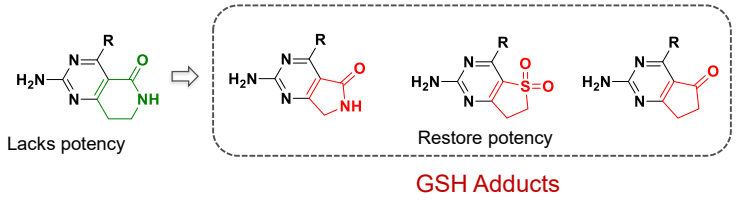
Case Study 3

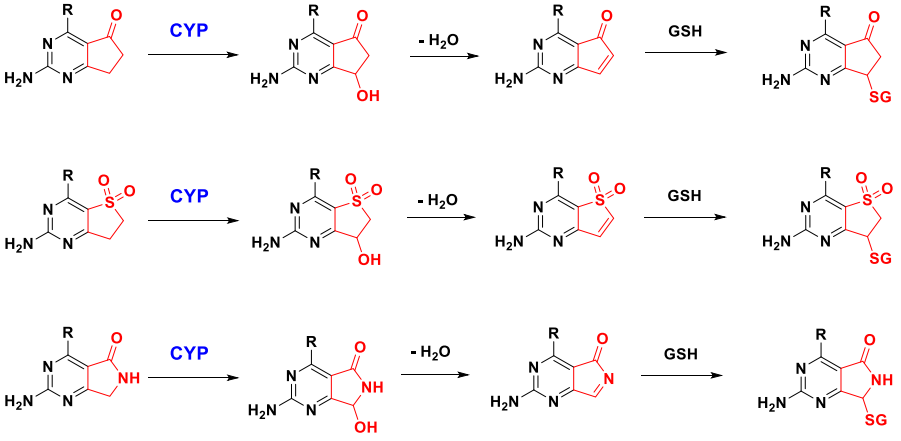
- Lead series is a substrate of aldehyde oxidase (AO)



- Block AO metabolism

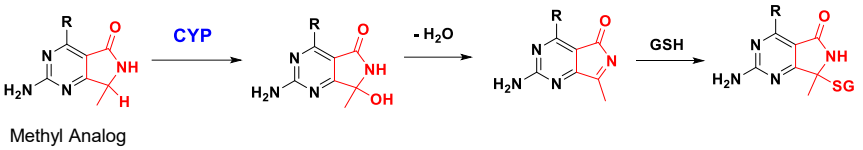


GSH Adducts Formed Through Common Mechanism

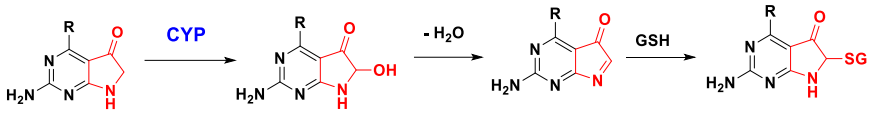


Efforts to Address GSH Adducts

- Unsuccessful attempts



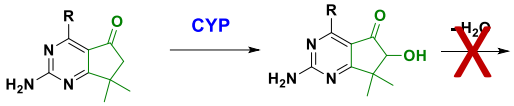
Methyl Analog



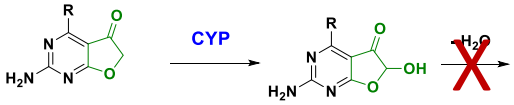
Positional Isomer

Efforts to Address GSH Adducts

- Successful Attempts

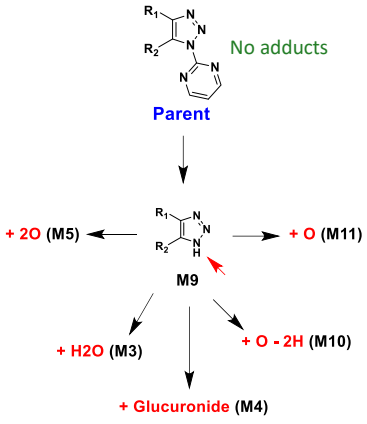


No adducts

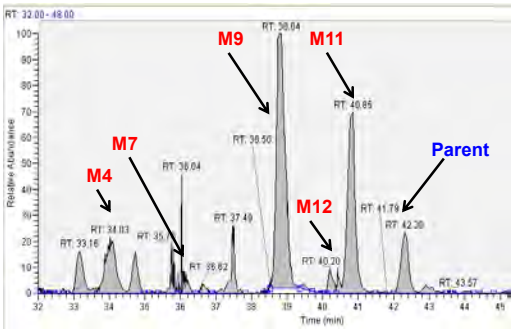


No adducts

Case Study 4

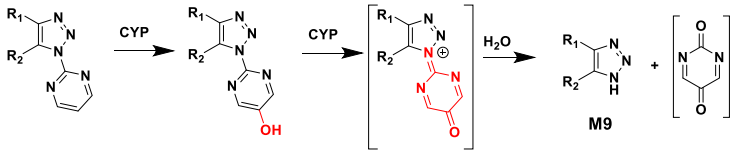


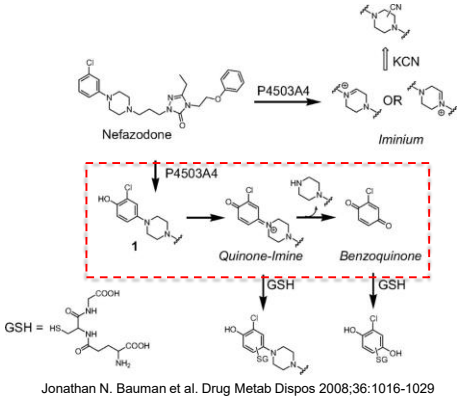
MS XIC of Rat Bile Sample



- Extensive metabolism from rat BDC study despite limited turnover in vitro
- Major metabolic route involves dearylation of pyrimidine

Dearylation Pathway Associated with Liver Injury



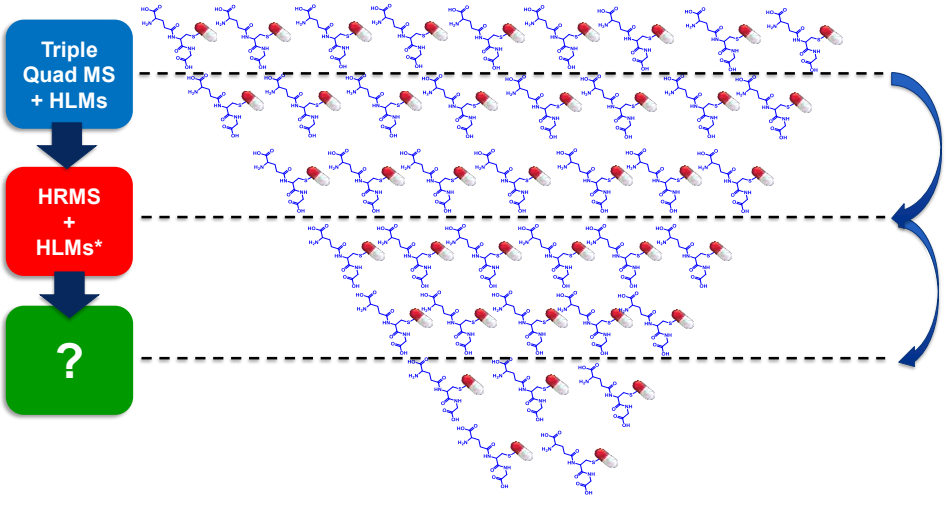


Jonathan N. Bauman et al. Drug Metab Dispos 2008;36:1016-1029

- Hepatotoxicity in rat
- Analogs that block dearylation pathway are safe

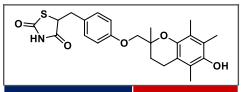
Trapping Assays are Intended for Hazard Identification

Trapping Assays are Intended for Hazard Identification

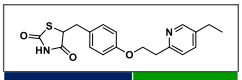


- Detection threshold ≠ safety threshold, rather detection threshold = hazard potential

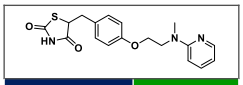
Adduct Detection Itself is Not a Predictor of Toxicity



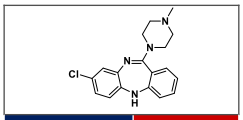
Troglitazone	DILI Positive
GSH HLM Trapping	Positive
CN HLM Trapping	Positive
Daily Dose	200 - 600 mg



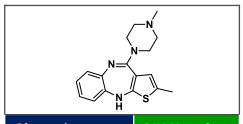
Pioglitazone	DILI Negative
GSH HLM Trapping	Positive
CN HLM Trapping	Negative
Daily Dose	30 mg



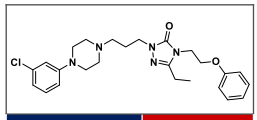
Rosiglitazone	DILI Negative
GSH HLM Trapping	Positive
CN HLM Trapping	Negative
Daily Dose	8 mg



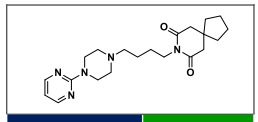
Clozapine	DILI Positive
GSH HLM Trapping	Positive
CN HLM Trapping	Positive
Daily Dose	> 300 mg



Olanzapine	DILI Negative
GSH HLM Trapping	Positive
CN HLM Trapping	Positive
Daily Dose	10 mg



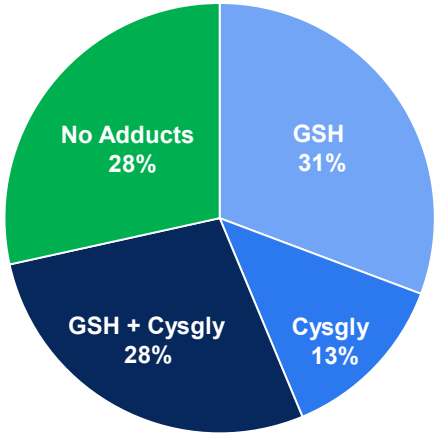
Nefazodone	DILI Positive
GSH HLM Trapping	Positive
CN HLM Trapping	Positive
Daily Dose	600 mg



Buspirone	DILI Negative
GSH HLM Trapping	Positive
CN HLM Trapping	Positive
Daily Dose	60 mg

Adduct Detection is a Frequent Occurrence

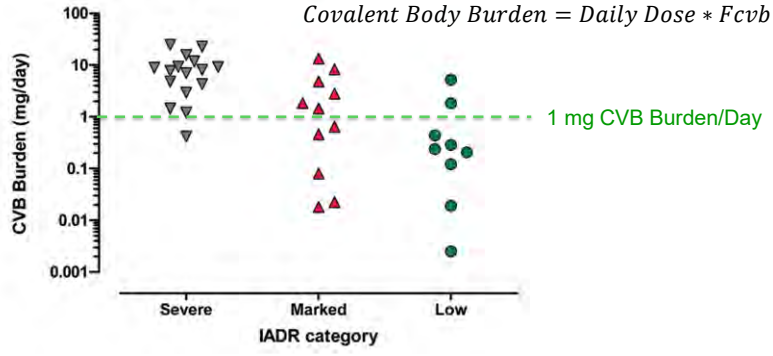
GSH Human Liver Microsomal Trapping
(Janssen - La Jolla)



N = 485 Compounds

Covalent Body Burden Allows for Further Risk Assessment

$$Covalent\ Body\ Burden = Daily\ Dose * Fcvb * Fa * Fg$$



- By determining the extent of covalent binding, a dose threshold associated with the 1 mg/ day body burden can be determined to influence decision-making

Thompson et al, *Chem Res Toxicol*, 2012

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Evolving DILI Landscape

- **Assembling the DILI Puzzle Pieces**
  - Integrated In Vitro Hazard Matrix – Thompson et al. *Chem Res Toxicol* 2012
  - Bayesian Machine Learning – Williams DP et al. *Chem Res Toxicol* 2020
  - Hepatic Risk Matrix – Aleo et al. *Chem Res Toxicol* 2020

- **Biological Signatures to Reactive Drug Metabolism**
  - Rat Liver Transcriptional Response – Monroe JJ et al. *Toxicol Sci* 2020
  - In Vitro Liver Model Transcriptomic Signature – Kang W et al. *Toxicol Sci* 2020

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Conclusions

- Reactive metabolism is one of multiple risk factors of DILI
- Test models and our detection methods will continue to advance
- Preventing reactive metabolism is challenging but surmountable
- Solutions possible with mechanistic insight and program commitment
- Science needed to guide model application and its translational value
- Contextualizing reactivity to broader risk assessment highly desirable
- Successful Discovery efforts aide reducing potential Development risks

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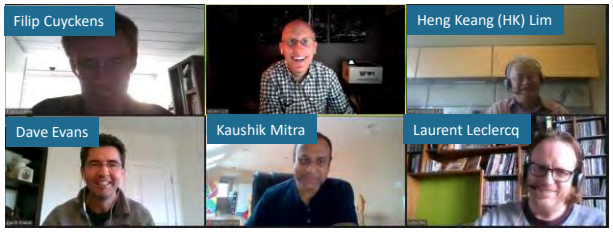
Acknowledgements

Janssen R&D Global Biotransformation Core Team



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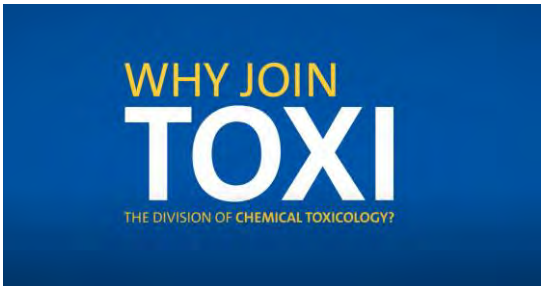

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THE CONTRIUBUTION OF MITOCHONDRIAL TOXICITY TO DRUG INDUCED LIVER INJURY

Yvonne Will, Ph.D.
Predictive, Investigative and Translational Toxicology

Janssen LLC, La Jolla, CA
ywill@its.jnj.com

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OUTLINE

✓ **The Business Case**

- Mitochondrial function and dysfunction
- Examples of Drugs and their mitochondrial targets
- Assays to detect mitochondrial dysfunction in vitro and ex vivo
- Animal models to study mitochondrial toxicity
- How do we avoid mitochondrial toxicity?
- Multifactorial toxicity
- Biomarker approaches
- Summary

40 Drugs have been attrited from the Market due to hepatotoxicity over the past 20 years

- At least 13 of those have been reported to exhibit mitochondrial toxicity

- Nefazodone, Benzarone, amineptine, nialamide, sitaxentan, dilevalol, troglitazone, tolcapone, pirprofen, alpidem, bromfenac,phenformin, buformin, trovafloxacin

Selected drugs associated with idiosyncratic DILI that exhibit a clear mitochondrial hazard

Drug	Mitochondrial liability in hepatocytes	Mitochondrial liability in other cell types
Troglitazone	Bedoucha et al. (2001); Haskins et al. (2001); Tirmenstein et al. (2002); Narayanan et al. (2003); Shishido et al. (2003); Bova et al. (2005); Masubuchi et al. (2006); Ong et al. (in press)	Atarod and Kehrer (2004); Konrad et al. (2005)
Diclofenac	Petrescu and Tarba (1997); Bort et al. (1998); Masubuchi et al. (1998); Masubuchi et al. (1999); Masubuchi et al. (2000); Masubuchi et al. (2003); Gomez-Lechon et al. (2003a); Gomez-Lechon et al. (2003b); Lim et al. (2006)	Mingatto et al. (1996); Uyemura et al. (1997); Pigoso et al. (1998); Moreno-Sanchez et al. (1999); Krause et al. (2003); Inoue et al. (2004); Taib et al. (2004)
Nimesulide	Mingatto et al. (2000); Caparroz-Assef et al. (2001); Mingatto et al. (2002); Tay et al. (2005); Ong et al. (2006)	Moreno-Sanchez et al. (1999)
Mefenamic acid	McDougall et al. (1983); Masubuchi et al. (2000)	Mingatto et al. (1996); Uyemura et al. (1997); Pigoso et al. (1998)
Tolcapone	Haasio et al. (2002a,b,c)	Korlipara et al. (2004)
Valproic acid	Bjorge and Baillie (1991); Keller et al. (1992); Ponchaut et al. (1992); Tang et al. (1995); Trost and Lemasters (1996); Sobaniec-Lotowska (1997); Tong et al. (2005)	Melegh and Trombitas (1997); Kawagoe et al. (2002)
Leflunomide	Spodnik et al. (2002)	
Amiodarone	Fromenty et al. (1990); Berson et al. (1998); Spaniol et al. (2001); Kaufmann et al. (2005)	Fromenty et al. (1993); Varbiro et al. (2003)
Trovafloxacin	Liguori et al. (2005)	
Simvastatin	Velho et al. (2006)	Caffório et al. (2005); Westwood et al. (2005)
Perhexiline	Deschamps et al. (1994); Berson et al. (1998)	
Isoniazid	Schwab and Tuschl (2003); Chowdhury et al. (2006)	
Dantrolene	Darios et al. (2003); Munns et al. (2005)	
Sulindac	Leite et al. (2006)	Daouphars et al. (2005); Park et al. (2005); Sinicrope and Penington (2005)
Fialuridine	McKenzie et al. (1995); Horn et al. (1997); Lewis et al. (1997)	Semino-Mora et al. (1997)
Lamivudine	Note et al. (2003)	Divi et al. (in press)
Stavudine	Gaou et al. (2001); Gerschenson et al. (2001); Pace et al. (2003); Velsor et al. (2004)	Lopez et al. (2004); Divi et al. (2005)

Boelsterli & Lim. Mitochondrial abnormalities--a link to idiosyncratic drug hepatotoxicity? *Toxicol Appl Pharmacol* 220:92-107, 2007 . With permission

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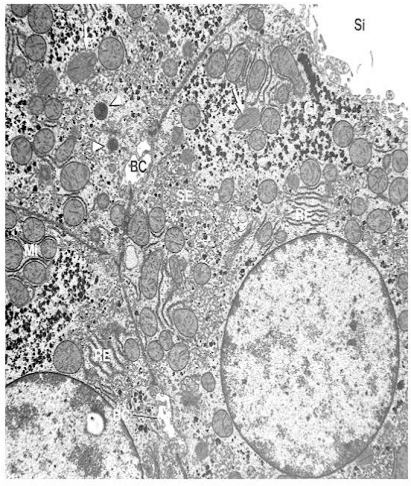
Function of Mitochondria



- Oxidative phosphorylation
- Steroid synthesis
- Fatty acid β-oxidation
- Heme synthesis
- Ca^{2+} homeostasis
- Urea cycle
- Steroid synthesis
- Apoptosis

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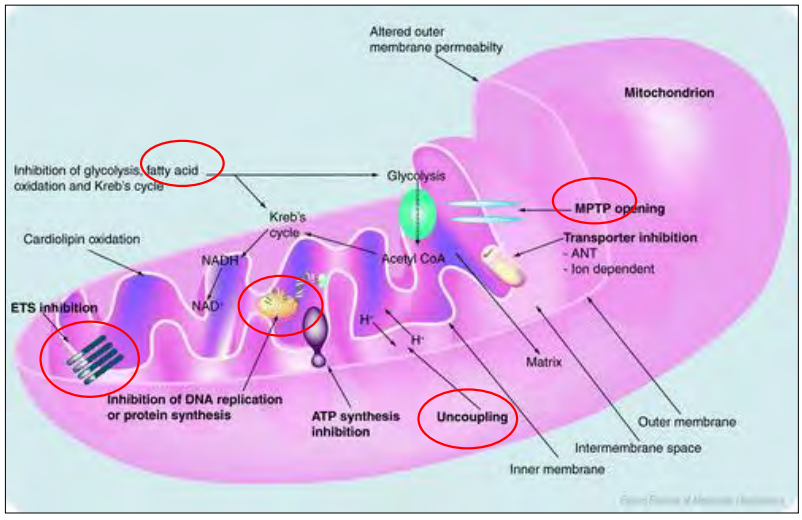
Many different mechanisms leads to mitochondrial dysfunction



Dykens JA, Marroquin LD, Will Y. Strategies to reduce late-stage drug attrition due to mitochondrial toxicity. *Expert Rev Mol Diagnostics* 7,161-75 (2007).

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DILI Drugs and their Mitochondrial Targets

FA-oxidation

- Amineptin
- Amiodarone
- Ibuprofen
- Perhexillin
- Tamoxifen
- Valproate

MPT induction

- Alpidem
- Diclofenac
- Nimesulide
- Troglitazone
- Valproate

ETC/delta Psi

- Amiodarone
- Nefazodone
- Tamoxifen
- Troglitazone
- Perhexillin
- Tacrine
- Nimesulide

mtDNA

- Fialuridine
- NRTIs
- Tacrine
- Tamoxifen

Mitochondrial Dysfunction can Induce the Following Types of Liver Injury

- **Microvesicular steatosis**
  - Profound hypoglycemia and encephalopathy
- **Apoptosis/necrosis**
  - Cytolytic hepatitis
    - Liver failure
- **Mild mitochondrial inhibition**
  - Macrovesicular steatosis
    - Progress to fibrosis and cirrhosis

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Examples of drugs capable of inducing microvesicular steatosis.

Drug	Indication
Amineptine	Antidepressant drug
Amiodarone	Anti-anginal, anti-arrhythmic drug
Pirprofen, Ibuprofen	NSAIDs
Aspirin	NSAID
Fialuridine	Antiviral (anti-HBV) drug
NRTIs	Antiretroviral (ant-HIV) drug
Panadiplon	Anxiolytic drug
Perhexiline	Anti-anginal drug
Tetracyclin	Antibiotic
Tianeptine	Antidepressant drug
Valproic acid	Antiepileptic drug

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Drugs capable of inducing microvacuolar steatosis and steatohepatitis.

Drug	Indication
Amiodarone	Anti-anginal, anti-arrhythmic drug
Irinotecan	Antineoplastic drug (colorectal cancer)
Methotrexate	Antipsoriatic, anti-rheumatoid drug
NRTIsb (AZT, ddI, d4T)	Antiretroviral (anti-HIV) drug
Perhexiline	Anti-anginal drug
Tamoxifen	Antineoplastic drug (breast cancer)
Toremifene	Antineoplastic drug (breast cancer)

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In vitro detection drug-induced mitochondrial dysfunction.

Isolated liver mitochondria

- Assessment of fatty acid oxidation (FAO) with radiolabelled fatty acids or using respiratory screening technology (Rogers et al., Curr Protoc Toxicol. 2014 May 27;60:25.3.1-25.3.19)
- Measurement of oxygen consumption with different substrates (Hynes J, et al., Methods Mol Biol. 2012;810:59-72)
- Measurement of mitochondrial respiratory chain complex activities (Nadanaciva et al., Toxicol In Vitro. 2007 Aug;21(5):902-11.
- Determination of mitochondrial transmembrane potential with a tetraphenylphosphonium chloride selective electrode, or by flow cytometry/imaging with a fluorescent probe (Billis et al., Curr Protoc Toxicol. 2014 Feb 19;59:25.1.1-25.1.14)
- Assessment of mitochondrial permeability transition pore (MPT) opening by spectrophotometry (Marroquin et al., Curr Protoc Toxicol. 2014 May 27;60:25.4.1-25.4.17)

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In vitro detection drug-induced mitochondrial dysfunction.

In hepatic-like cells (HepG2, HepRG) or primary hepatocytes, Hepatopac, liver chip, etc.

- Coloration with oil red O (for the detection of neutral lipids)
- Measurement of lactic acid/ALT in the incubation medium
- Assessment of FAO with radiolabelled fatty acids or by respiration (see previous slide)
- Measurement of oxygen consumption (see previous slide)
- Determination of mitochondrial transmembrane potential by flow cytometry or High Content imaging (see previous slide)
- Assessment of mtDNA levels by PCR (Venegas and Halberg, Methods Mol Biol. 2012;837:327-35.)
- High Content Imaging of lipids, membrane potential, ROS, apoptosis etc (Xu et al., Toxicol Sci. 2008 Sep;105(1):97-105)

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Examples of *in vivo* or *ex vivo* investigations which can be performed to detect drug-induced mitochondrial dysfunction.

- **Plasma biochemistry:** lactate, ketone bodies, GLDH
- **Urine biochemistry:** acyl-carnitine and acyl-glycine derivatives
- **Histopath** (with oil red O and haematoxylin-eosin staining) **and EM**
- **Assessment of whole-body fatty acid oxidation** (FAO) **after administration of ^{14}C-labelled fatty acids**
- **Investigations on liver mitochondria or hepatocytes isolated from treated animal**
  - As described on previous two slides
- **Investigations on liver homogenates prepared from treated animals:**
  - assessment of mtDNA levels and/or activities of different mitochondrial respiratory chain complexes, immunoblot analysis of selected MRC or FAO polypeptides

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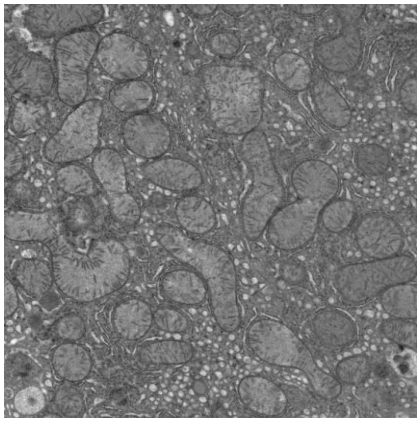
Mice/Rats are resistant to many mitochondrial toxicants

- **High rates of drug biotransformation/elimination**
- **High electrophile/antioxidant defense capacity**
  - Hepatic GSH content → rodents > humans (~2x)
  - Hepatic GST activity → rodents >> humans (10-20x)
- **Heteroplasmy**
  - rapid turnover of mitochondria
- **Mitochondrial threshold effects**
  - ATP levels
  - Apoptosis
  - mtDNA damage



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The *Jvs*$^{+/-}$ mouse model

- Mutation in gene coding for OCTN2 (carnitine transporter)

→ impaired renal absorption of carnitine,

→ systemic carnitine deficiency (~50%)

Phenotype: liver steatosis, hypoglycemia, cardiac hypertrophy

Mitochondrial abnormalities in muscle

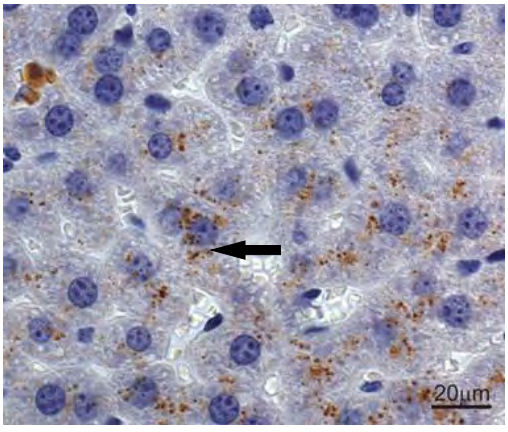
Jvs$^{-/-}$ genotype is lethal without carnitine replacement

VPA (2.5g/kg/d, p.o., x 14 days)

- Increased serum markers for liver injury
- (AST and ALP activity)
- Microvesicular steatosis
- Caspase-3 activation → apoptosis

→Carnitine deficiency may be a risk factor for VPA hepatotoxicity!

Knapp *et al.* (2008)



Anti-caspase-3 (cleaved) Ab

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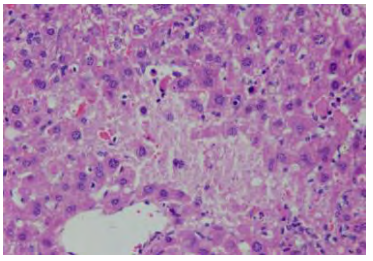
The *Sod2*$^{+/-}$ mouse model

Flutamide

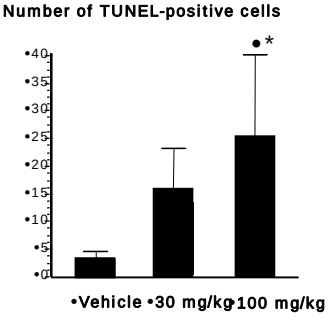
Sod2$^{+/-}$ or *Sod2*$^{+/+}$ mice (wt)
0, 30, or 100 mg/kg/day, ip
x 2 wk or 4 wk

hepatic necrosis (FLU 100 mg/kg/d, 4 wk)



↓ Aconitase activity
↓ Complex I activity
↓ mtDNA-encoded subunits of complex I and III

No changes (vs. vehicle control) in wild-type mice !
No toxicity after bicalutamide (non-DILI)



Kashimshetty *et al. (*2009)

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How predictive are these animal models for drug development?

- Need to be validated (w/ negative comparators)
- Require great expenditure in time and money
- Mitochondrial changes frequent, but do not always translate into organ damage- multifactorial toxicity? Idiosyncracy?
- Can they assist in the search for early biomarkers that could be translatable to "normal" rats and mice and to humans?
- Biomarkers need to be non-invasive (methionine breath test, GLDH)

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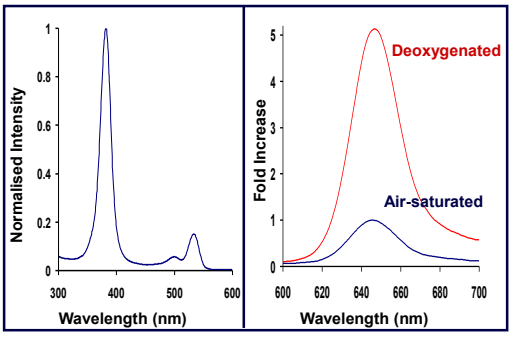
Assays to Detect Mitochondrial Toxicity

- Assay for measuring Oxygen consumption of isolated mitochondria.
- Cell viability assay in (a) Glucose medium, (b) Galactose medium.
- Assay for measuring Oxygen consumption and extracellular acidification of cells.
- Assays for measuring changes in mtDNA and mtDNA-encoded protein levels in cells.

Oxygen consumption Measurement in Isolated Mitochondria is a surrogate for ATP production



- Phosphorescent
- Water-soluble
- Cell non-invasive
- Non-cytotoxic
- Stable
- Time resolved or prompt
- Compatible with any reader
- Large stoke shift allows for high signal to noise ratio
- multiplex with “green dyes”

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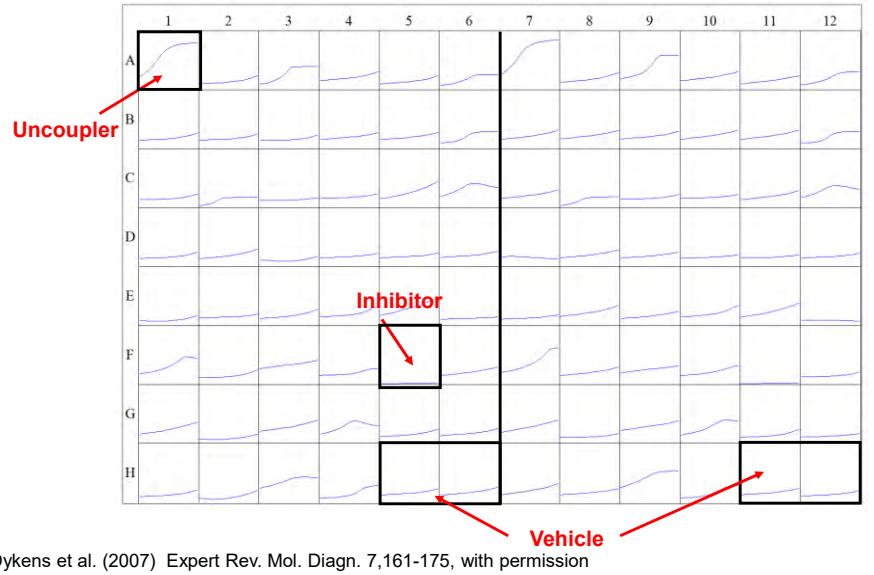
Output of Fluorescent Data from the Oxygen-Sensing Probe with Isolated Mitochondria

Basal respiration



Dykens et al. (2007) Expert Rev. Mol. Diagn. 7,161-175, with permission

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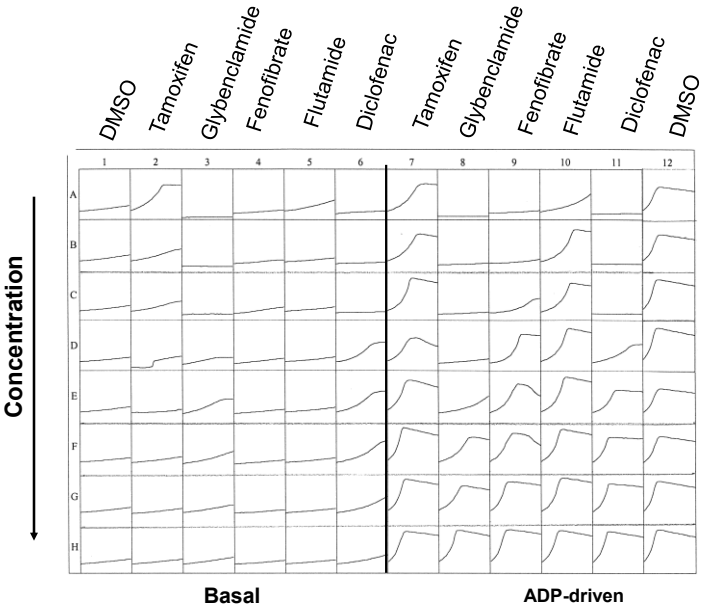
Dose Response Curves can Easily be Generated



Hynes et al. (2006) Toxicol. Sci. 92, 86-200

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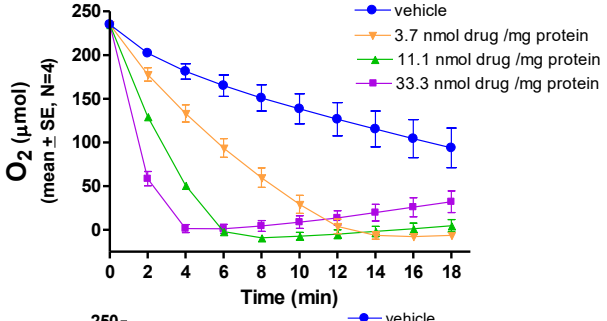
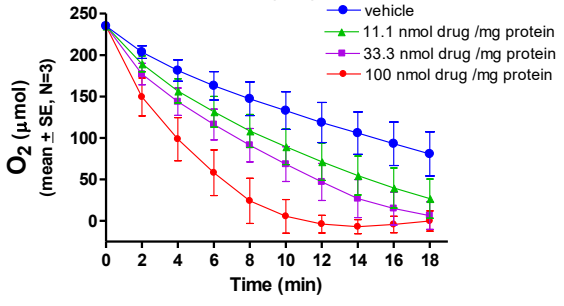
Some Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) Uncouple Electron Transport in Isolated Rat Liver Mitochondria

Nimesulide



Sudoxicam

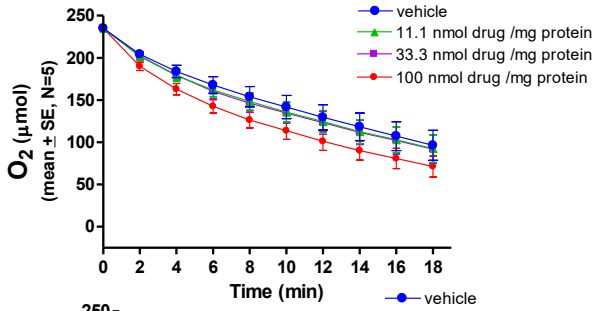


Nadanaciva, unpublished

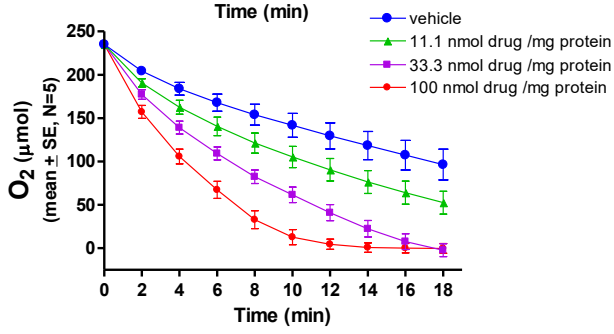
90

Sulindac Sulfide, the Reactive Metabolite of the NSAID Sulindac, Causes Uncoupling of Electron Transport in Isolated Rat Liver Mitochondria

Sulindac



Sulindac sulfide



Nadanaciva, unpublished

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Summary: Oxygen Consumption of Isolated Mitochondria

Values:

- Identifies inhibitors and uncouplers of the electron transport chain
- High-throughput; highly reproducible; easy to use
- Can rank order compounds within a series for their mitochondrial toxicity effects
- May be used to identify structure-activity-relationships

Limitations:

- Can potentially overestimate toxicity since the isolated organelle is being used
- Identifies only immediate (acute) effects; may need to pre-incubate mitochondria with drug
- Does not take into account conversion of parent drug → reactive/inactive metabolites

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Circumventing the *Crabtree Effect*: **The "Glucose-Galactose" Model**

Crabtree Effect (1929): inhibition of respiration by elevated glucose.

Warburg Effect (1929): aerobic glycolysis yields lactate despite competent mitochondria.

Contemporary cell culture often uses 25mM glucose media (5X physiological!)

Transformed cells are characterized by low rates of O_2 consumption & resistance to mitotoxicants.

Marroquin et al. (2007) Toxicol. Sci., 97, 539-547

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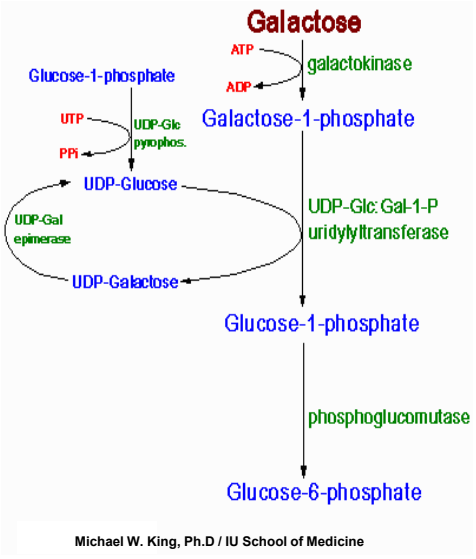
Galactose in Glycolysis Yields Little ATP



Net yield ATP = 0.4 mol/mol gal*

* Reitzer et al., JBC, 254: 2669, 1979.

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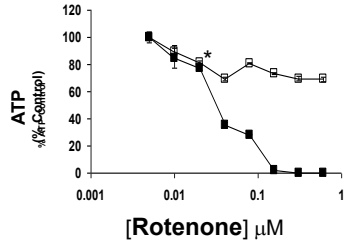
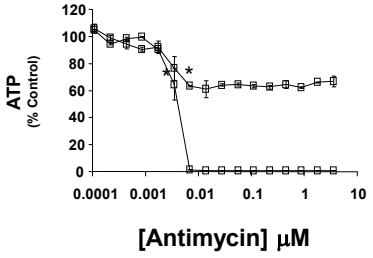
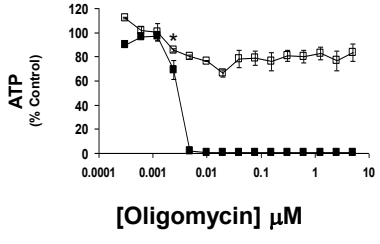
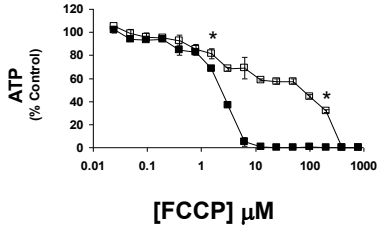
Cells Grown in Galactose Become Susceptible to Mitochondrial Toxicants









Marroquin et al. (2007) Toxicol. Sci., 97, 539-547

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Industry use of HepG2 cells to detect Mitotoxicity

Questionnaire to pharma partners on use of HepG2 cells for preclinical screens

8/8 used HepG2 screens

6/8 routinely use for mitotox testing

All 6/8 use ***galactose media*** for mitotox testing

Information used to rank compounds, inform structure activity relationships and/or inform drug design.

Circumventing the Crabtree Effect: Replacing Media Glucose with Galactose Increases Susceptibility of HepG2 Cells to Mitochondrial Toxicants

Lisa D. Marroquin,*,‡ James Hynes,†,‡ James A. Dykens,* Joseph D. Jamieson,* and Yvonne Will*,†

*Pfizer DSRD, 10646 Science Center Drive, San Diego, California 92121; and †Laxcel Biosciences Ltd, BioInnovation Centre, BioTransfer Unit, University College Cork, Cork, Ireland

Evidence of mitochondrial dysfunction through cell death

Courtesy of Amy Mercer, MRC, UK

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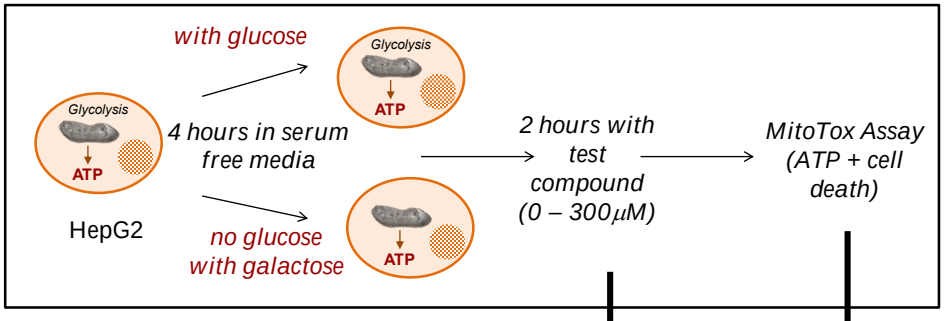
Acute Metabolic Switch Model: HepG2 cells



Short exposure time and dual assay allows examination of the role of mitochondrial dysfunction in the absence of cell death.

Courtesy of Amy Mercer

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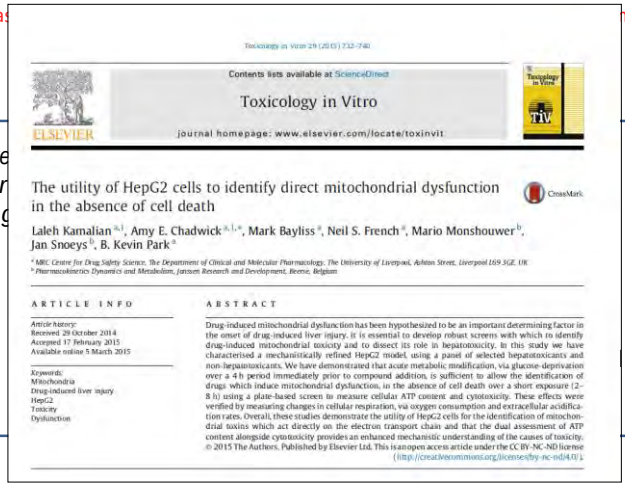
Recommendation to Pharma

The dual as mechanistic

Sensitive first-tier screening



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Toxicology in Vitro

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The utility of HepG2 cells to identify direct mitochondrial dysfunction in the absence of cell death

Laleh Kamalian [a,1], Amy E. Chadwick [a,1,*], Mark Bayliss [a], Neil S. French [a], Mario Monshouwer [b], Jan Snoeys [b], B. Kevin Park [a]

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ABSTRACT

Drug-induced mitochondrial dysfunction has been hypothesized to be an important determining factor in the onset of drug-induced liver injury. It is essential to develop robust screens with which to identify drug-induced mitochondrial toxicity and to dissect its role in hepatotoxicity. In this study we have characterised a mechanistically refined HepG2 model, using a panel of selected hepatotoxicants and non-hepatotoxicants. We have demonstrated that acute metabolic modification, via glucose-deprivation over a 4 h period immediately prior to compound addition, is sufficient to allow the identification of drugs which induce mitochondrial dysfunction, in the absence of cell death over a short exposure (2–8 h) using a plate-based screen to measure cellular ATP content and cytotoxicity. These effects were verified by measuring changes in cellular respiration, via oxygen consumption and extracellular acidification rates. Overall, these studies demonstrate the utility of HepG2 cells for the identification of mitochondrial toxins which act directly on the electron transport chain and that the dual assessment of ATP content alongside cytotoxicity provides an enhanced mechanistic understanding of the causes of toxicity.

Limitations of the screen include detecting alternative mechanisms of mitochondrial dysfunction or mitotoxicity induced via reactive metabolites.

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OUTLINE

- The Business Case
- Mitochondrial function and dysfunction
- Examples of Drugs and their mitochondrial targets
- Assays to detect mitochondrial dysfunction in vitro and ex vivo
- Animal models to study mitochondrial toxicity
- How do we avoid mitochondrial toxicity?
- ✓ Multifactorial toxicity
- Biomarkers
- Summary

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TOXICOLOGICAL SCIENCES 103(2), 335–345 (2008)
doi:10.1093/toxsci/kfn056
Advance Access publication March 15, 2008

In Vitro Assessment of Mitochondrial Dysfunction and Cytotoxicity of Nefazodone, Trazodone, and Buspirone

James A. Dykens,* Joseph D. Jamieson,† Lisa D. Marroquin,† Sashi Nadanaciva,‡ Jinghai J. Xu,§ Margaret C. Dunn,§ Arthur R. Smith,§ and Yvonne Will¶,1

*Drug Safety Research and Development, Pfizer, Inc., Sandwich, UK CT13/NJ; †Drug Safety Research and Development, Pfizer, Inc., San Diego, California 92121; ‡MitoScience, Inc., Eugene, Oregon 97403; §Systems Biology, Pfizer Research Technology Center, Pfizer, Inc., Cambridge, Massachusetts 02139; and ¶Exploratory Safety Differentiation, Pfizer, Inc., Eastern Point, Groton, Connecticut 06340

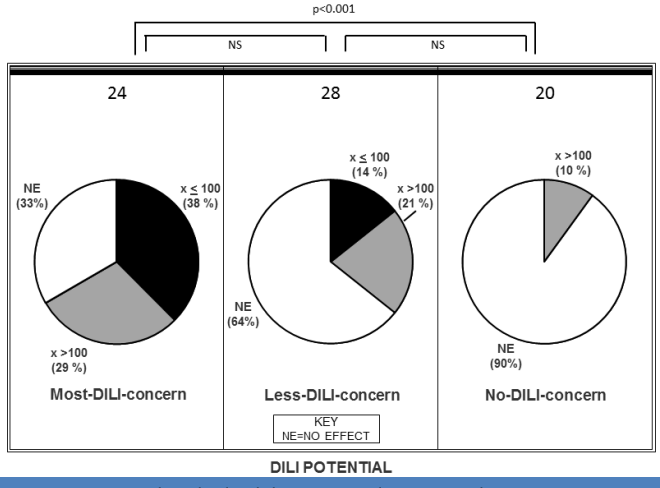
TOXICOLOGICAL SCIENCES 90(2), 451–459 (2006)
doi:10.1093/toxsci/kfj095
Advance Access publication January 12, 2006

Inhibition of Hepatobiliary Transport as a Predictive Method for Clinical Hepatotoxicity of Nefazodone

Seva E. Kostrubsky,*,1 Stephen C. Strom,† Amit S. Kalgutkar,‡ Shaila Kulkarni,* James Atherton,§ Rouchelle Mireles,‡ Bo Feng,‡ Raylene Kubik,* Janean Hanson,* Ellen Urda,* and Abdul E. Mutlib§

*Departments of Safety Science and §Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Ann Arbor, Michigan 48105 and ‡Groton, Connecticut 06340; and the †Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15261

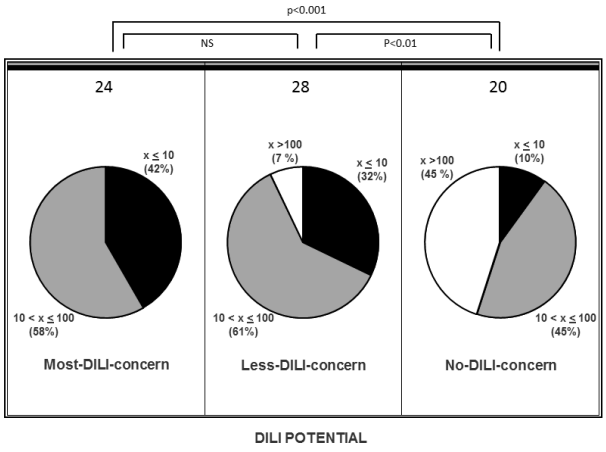
Intrinsic Potency of Mitochondrial Inhibition as Related to Clinical DILI Potential



- Relative potency as a mitochondrial inhibitor is evenly expressed in Most-DILI-concern compounds but is disproportionately distributed in No-DILI-concern category

Human drug-induced liver injury severity is highly associated to dual inhibition of liver mitochondrial function and bile salt export pump. Aleo MD[1], Luo Y, Swiss R, Bonin PD, Potter DM, Will Y. Hepatology. 2014 May 6.

Intrinsic Potency of Mitochondrial Inhibition as Related to Clinical DILI Potential



- Relative potency as a BSEP inhibitor is evenly expressed in Most-DILI-concern compounds but is disproportionately distributed in No-DILI-concern category

Human drug-induced liver injury severity is highly associated to dual inhibition of liver mitochondrial function and bile salt export pump. Aleo MD[1], Luo Y, Swiss R, Bonin PD, Potter DM, Will Y. Hepatology. 2014 May 6.

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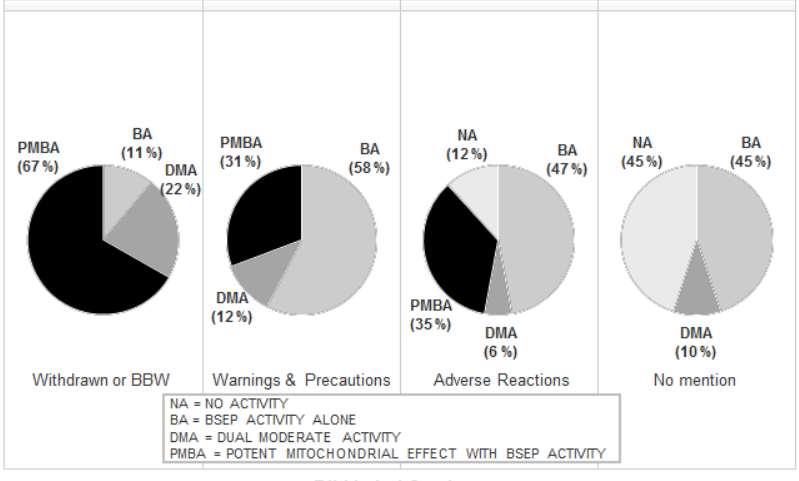
INTRINSIC BSEP AND MITOCHONDRIAL INHIBITORY LIABILITIES TIED TO FDA LABELS FOR LIVER INJURY



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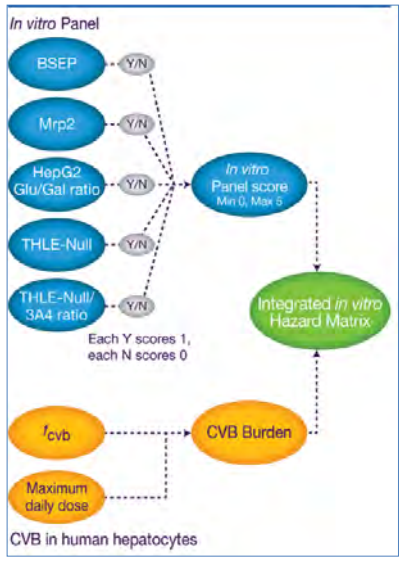
Known Risk Factors for Hepatic Injury

PATIENT RELATED FACTORS

- Underlying disease
- Co-medications (herbals, APAP) and concurrent exposures (including environmental and work-related)
- Age, diet and gender
- Physical activity
- Genetic acquired variability (e.g. in CYPs, transporters, HLA)
- Innate and adaptive immune response

DRUG RELATED FACTORS

- Overt Cytotoxicity
- Metabolism-related toxicity
  - Bioactivation and covalent binding to macromolecules
- Non-metabolism- related toxicity
  - Inhibitions of key cell functions (e.g. mitochondrial, lysosomal, biliary)
- Kinetics of the drug and its metabolites
- Tissue Exposure



Chem. Res. Toxicol. 2012, 25, 1616−1632

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- Animal models to study mitochondrial toxicity
- How do we avoid mitochondrial toxicity?
- Multifactorial toxicity

✓ Biomarkers

- Summary

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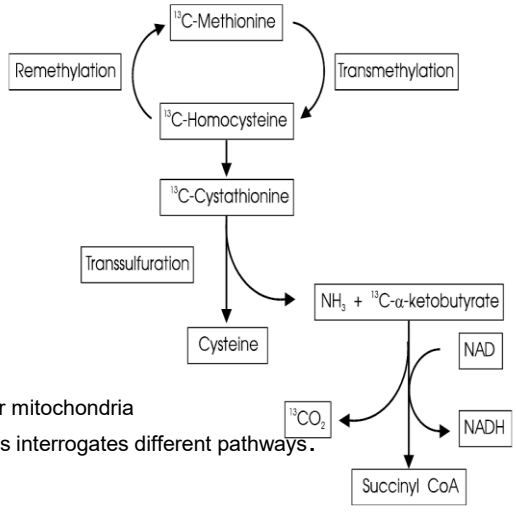
Serum Biomarkers

- The European Medicines Agency (EMEA) recommends the determination of lactate levels, as well as glutamate dehydrogenase (GLDH) and ornithine carbamoyltransferase (OCT) activities
- GLDH and OCT are mitochondrial enzymes. An increased plasma activity of these enzymes reflects structural damage to the mitochondria and cell membrane, leading to the leakage of these enzymes into the plasma
- OCT is particularly expressed in the liver
- Therefore, a high plasma OCT activity can occur when mitochondrial damage has specifically caused liver injury
- Mitochondrial miRNA have recently been explored (Baumgart BR, Gray KL, Woicke J, Bunch RT, Sanderson TP, **Van Vleet** TR. Toxicol Appl Pharmacol. 2016 Dec 1;312:26-33)

Methionine Breath Test: A Stable Isotope Technique



Measure in exhales breath. Primarily liver mitochondria

^{13}C-methionine – labelled at different sites interrogates different pathways.

Summary

- Mitochondrial toxicity contributes to DILI
- Mitochondrial Toxicity can be detected using in vitro assays
- Most pharmaceutical companies have implemented mitochondrial toxicity testing early in the drug discovery process (MIP-DILI)
- Animals do not easily reveal mitochondrial toxicity
- non invasive human biomarkers need to be developed and utilized in the clinic

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Kevin Coe
Senior Principal Scientist, Janssen Pharmaceutical Companies of Johnson & Johnson

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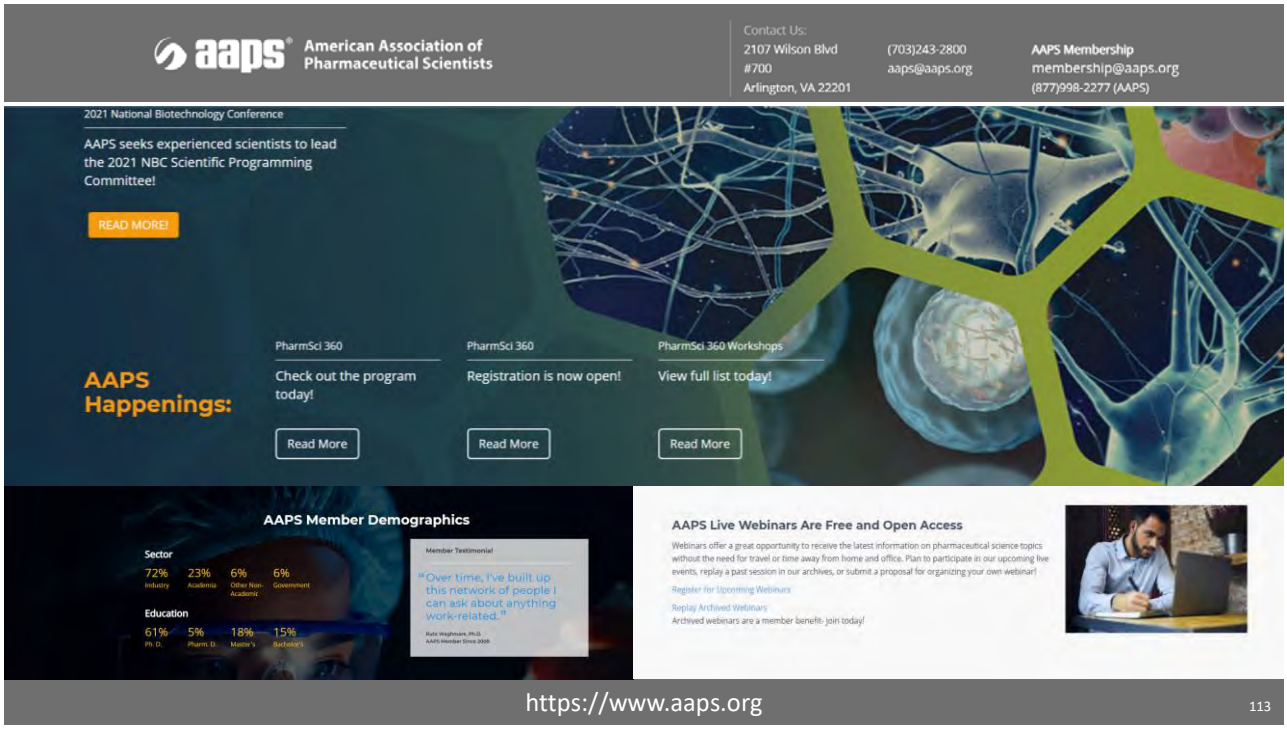

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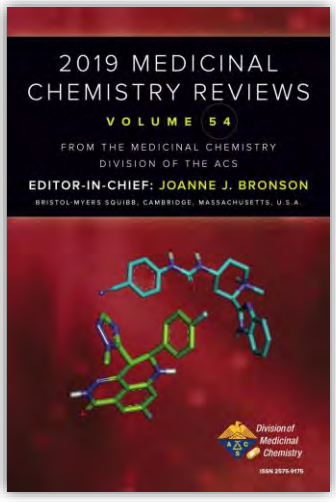

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