Non-CYP Drug Metabolism Pathways

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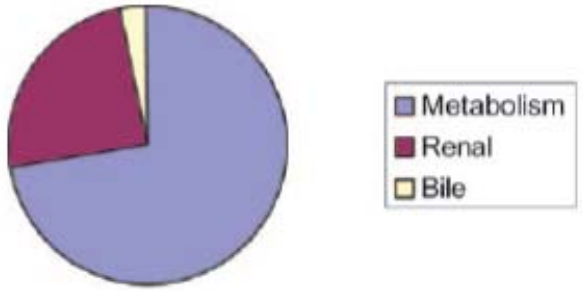
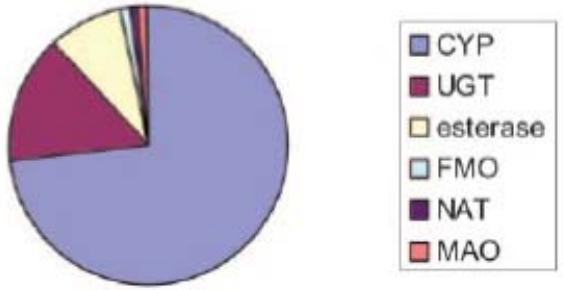
Importance of Non-CYP Pathways for Drug Clearance





Williams et al., DMD, 2004

Top 200 prescribed drugs in 2002

- Metabolism is predominant means of elimination
- CYP is major metabolic enzyme, followed by UGTs

- ~1/3 of drugs metabolized by non-CYP pathways
  - UGT
  - Esterase
  - Other phase 1 and phase 2 enzymes

- Current trend to design drugs with non-CYP metabolic pathways of elimination to avoid CYP DDI's

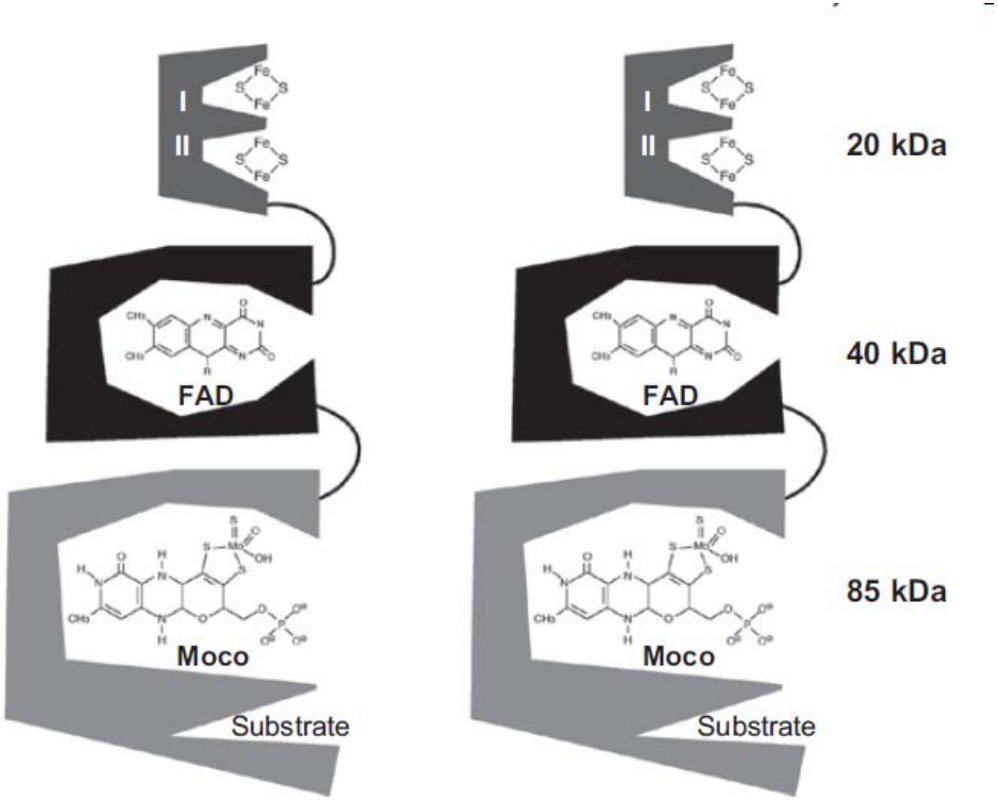
Today's Presentation

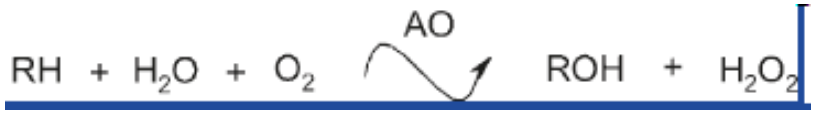
- Aldehyde oxidase
- Carboxylesterase
- UDP-glucuronidation

Aldehyde Oxidase

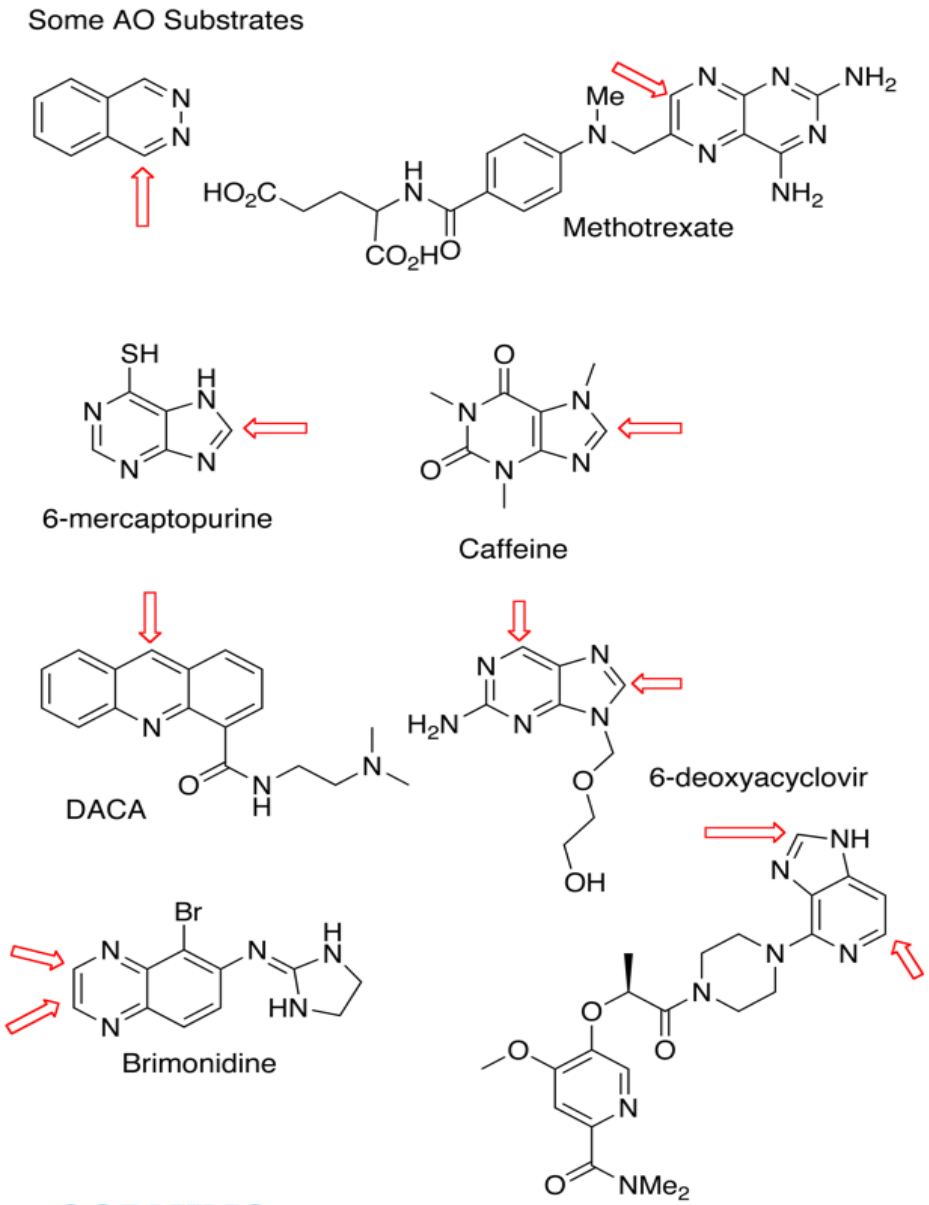
Aldehyde Oxidase Background

- Molybdo-flavoenzyme (MOFEs)
- Soluble, cytosolic enzyme
- Exists as a homo-dimer of two identical subunits, each ~150KD, each subunits contains the following:
  - 2Fe/2S centers
  - FAD
  - MoCo co-factor, which is near active site
- Oxidizes numerous drug molecules
  - **Aromatic Aza-heterocycles**
  - Aldehydes (aromatic)
  - Iminium ion intermediates
  - Reduction



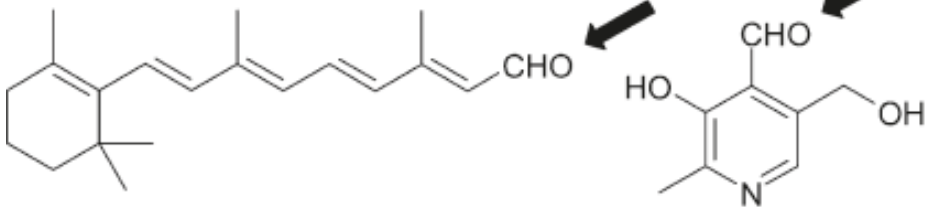


Aromatic Aza-heterocyle Substrates



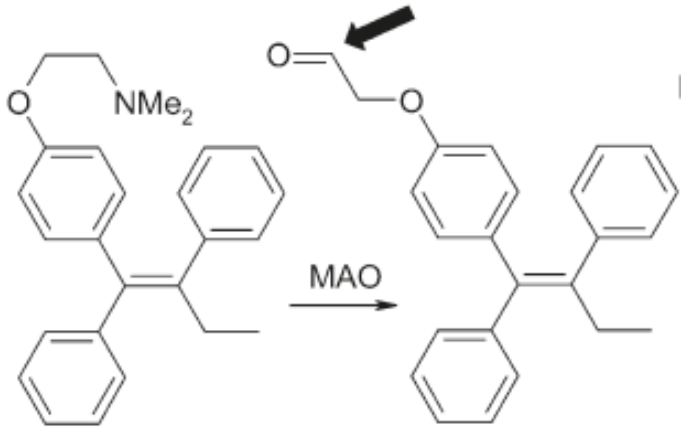
- Aromatic aza-heterocycles required in scaffold for targeting kinase inhibitors
- Introduction of nitrogen in aromatic ring of heterocycle reduces CYP activity
- Becomes better substrate for AO
- Hydroxylation (nucleophilic attach) of carbon adjacent to nitrogen

Aldehyde and Iminium Ion Substates

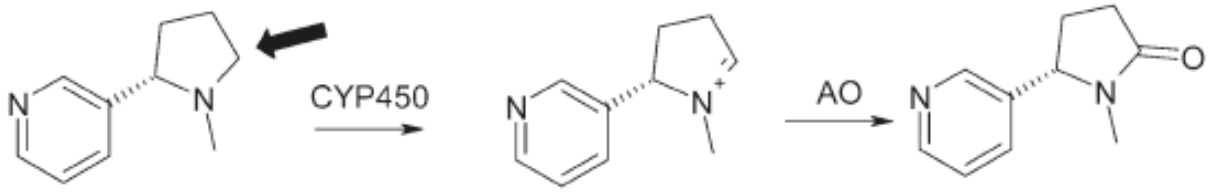


Vitamin A, Retinal

Vitamin B6



Tamoxifen



Nicotine

Cotinine

Tissue Distribution, Species Differences, Genes, Patient Variability

- AO is most abundant in the liver, also abundant in adrenal tissue
- Widespread distribution in other tissues (immunohistochemical studies): Skin, intestine, kidney, and lung have lower abundance/activity
  - Clearance for AO drugs > hepatic blood flow, suggesting possible involvement of extra-hepatic metabolism
- Large species difference: Human, monkey, guinea pig have highest activity, rat is intermediate, and dog is very low (absent) in AO activity
  - Strain differences observed in mice and rats
- In humans, AO is represented by a single gene product (AOX1), no isoforms
  - Rats and mice have 4 functional genes, monkey (rhesus) has 3 genes
  - Monkey, guinea pig, rat, and mouse all express the human ortholog, AOX1, dog does not have AOX1
- AO shows high patient-to-patient variability in activity (>40-fold reported)
  - Several SNPs have been identified which could be contributing factor
  - Lower variability reported for protein levels (3- to 4-fold via LC/MS [Fu, DMD, 2013]): co-factor depletion, SNPs, and tissue damage during processing

Aldehyde Oxidase vs. CYP Drug Metabolism

- AO is present in cytosol (CYP is in microsome)
  - Systems to study AO: S9, cytosol, hepatocytes
- AO does not require a soluble co-factor, e.g. NADPH
- H_2O is source of oxygen for AO-dependent drug oxidation, vs. O_2 for CYP
- AO prefers electron deficient carbons (nucleophilic attack of electrophilic carbons), vs. CYP, which prefers electron rich carbons
  - Reducing the electron density of a carbon in order to avoid CYP metabolism, can result in nucleophilic attack by AO

Importance of AO in human drug metabolism

- AO is typically not present in the “standard” metabolic stability assay using liver microsome.
- Chemical strategy to reduce P450 metabolism leads to alternative metabolic clearance mechanism, such as AO.
- That the AO pathway has been overlooked leads to clinical failures; higher-than-predicted clearance or toxicological outcomes in human (Diamond, et al., 2010),

Proportion of AO substrates in current compound collection (Pryde, J. Med. Chem, 2010)

- Authors analyzed several drug data bases for potential substrates of AO.
- Analysis based on known structural requirements of AO substrates (e.g. aromatic heterocycles).
- Conclusions:
  - Few drugs have gone to market that are AO substrates.
  - Drugs in current development pipeline are at greater risk of being AO substrates.
  - GPCR and Kinase targeted subsets showed the highest risk of AO metabolism.

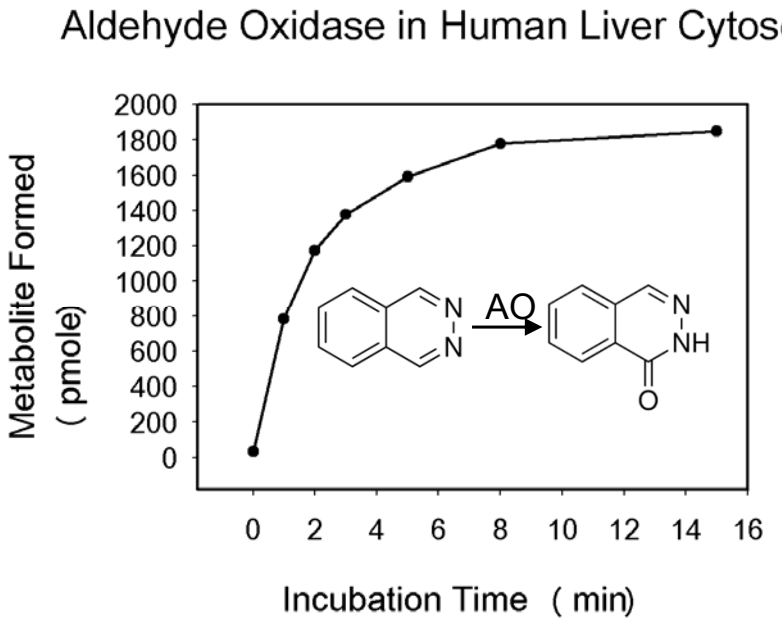
Drug Failures due to AO

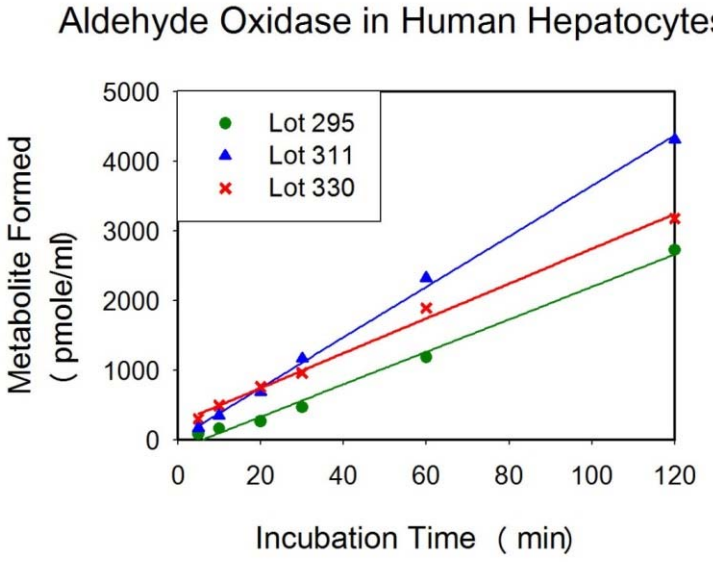
- FK3453: Parkinson's disease
  - Favorable PK in rat and dog
  - Good metabolic stability in rat, dog, and human HLM
  - Predicted human *in vivo* clearance: 1.3 mL/min/kg
  - Observed *in vivo* clearance: 3,000 mL/min/kg
  - Drug withdrawn from phase 1 trials
  - Using selective AO and CYP inhibitors, AO was identified as predominant pathway
- Other failures
  - SGX523: Cancer drug
  - P38 Kinase inhibitor: arthritis
- Common Theme
  - Inappropriate preclinical species: rat, dog
  - Emphasis on HLM (i.e., CYPs)

In Vitro Systems for Studying Aldehyde Oxidase

- Hepatocytes, S9, and cytosol correlate well with one another
- *In vitro* scaled intrinsic clearance underestimates *in vivo* clearance.
- Assay time-course linearity can be an issue with tissue fractions, hepatocytes show better linearity.
- Stability of AO in hepatocytes and tissue fractions seen as possible cause for patient variability and under prediction of drug clearance
  - Cryopreservation of hepatocytes has little effect on AO activity
  - AO in S9 and cytosol is stable to freeze/thaw (in-house data)
- Microsomes can be contaminated by cytosolic AO (it is important to “wash” microsome fraction with an extra high speed spin.)

AO Activity in Human Liver Cytosol

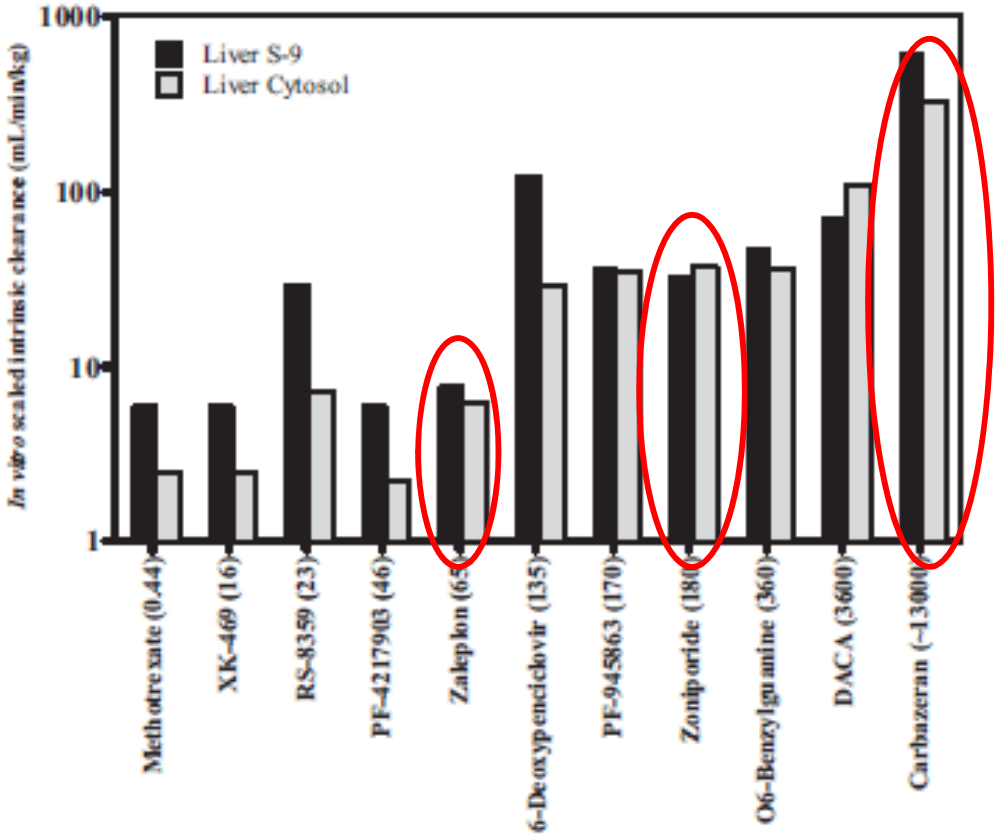




- Phthalazine is probe substrate of AO
- Short assay linearity in cytosol and rAO, but not in hepatocytes
- Reported by other groups for phthalazine
- Substrate-dependent
- Also reported for benzylaldehyde oxidation (Kitamura, *Life*, 1999), in same paper Methotrexate showed long linear time course.
- Product inhibition possible explanation

In Vitro Systems to Assess AO Activity
- S9, Cytosol, Hepatocytes (Zientek, DMD, 2010; Hutzler, DMD, 2012)

- Hepatocytes (pooled), S9, and cytosol under predict Cl
  - ~11-fold under prediction for tissue fractions, ~3-fold for hepatocytes
- S9 and Cytosol correlate well with each other
- Zientek, et.al., proposed "calibration method" for predicting *in vivo* clearance
  - Cl_{int} < Zaleplon: Low clearance
  - Zonipride: Moderate
  - Cl_{int} > Carbazeran: High clearance
- Under prediction may be due in part to extra-hepatic metabolism



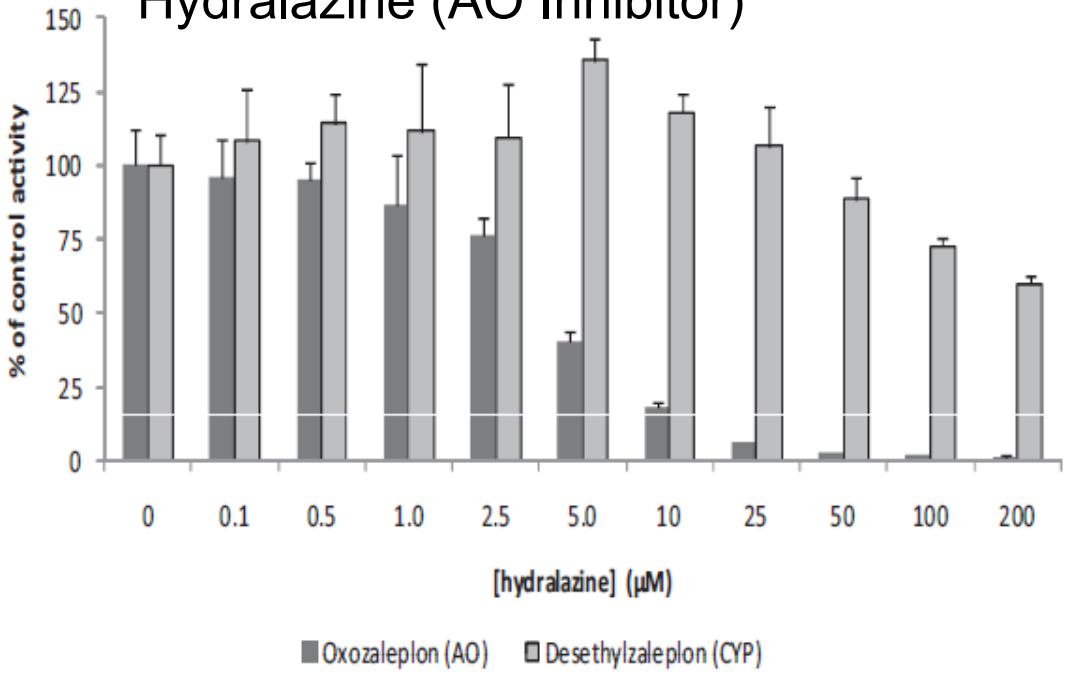
Aldehyde Oxidase Substrates and Inhibitors

- Drug substrates (Pryde, J. Med. Chem, 2010)
  - Zaleplon
  - Carbazeran
  - Methotrexate
  - Zoniporide
- Commonly used probe substrates
  - Phthalazine
  - Vanillin
- Inhibitors
  - Raloxifene (Obach, DMD, 2004), TDI
  - Hydralazine (Strelevitz, DMD, 2012), TDI

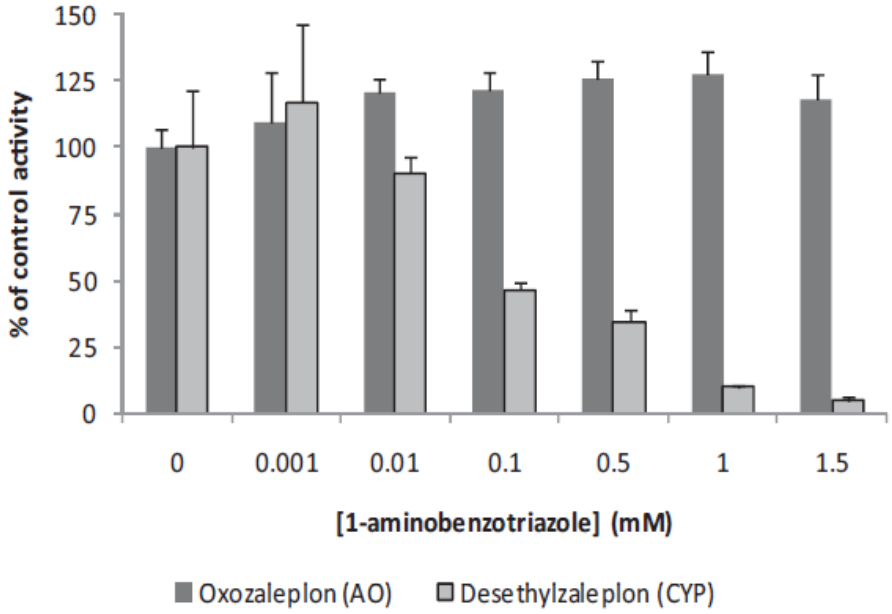
Determining Fraction Metabolized in Human Hepatocytes with TDI Hydalazine (Strelevitz, DMD, 2012)

- Zaleplon metabolized by CYP and AO pathways
  - P450 metabolite desethylzaleplon
  - AO metabolite oxozaleplon
- Hydralazine at 25 to 50 µM was selective for AO metabolite in hepatocytes
- Minor inhibition of CYPs (2D6 and 3A4) at 50 µM (tested in HLM)
- Hydalazine was non-cytotoxic to PHH at concentrations used in the study

Hydralazine (AO Inhibitor)

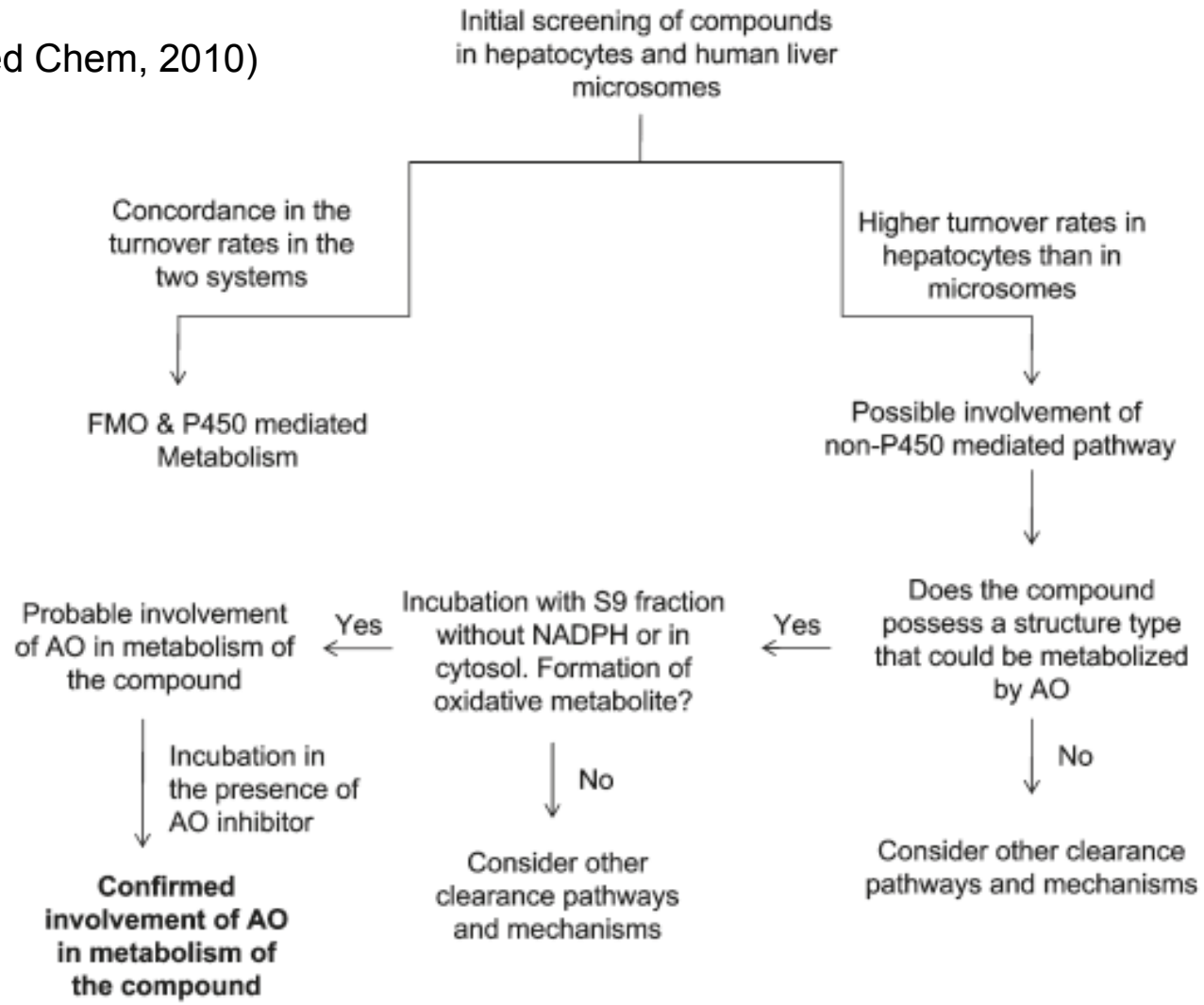


AMBT (CYP Inhibitor)



Aldehyde Oxidase Decision Tree

(Pryde, et. al, J Med Chem, 2010)



Decision tree to guide decision-making during the screening of potential AO substrates.

Conclusions: Aldehyde Oxidase

- As Pharma designs drugs away from CYP metabolic pathways, the likelihood of introducing an AO pathway tends to increase.
- Current compound libraries in development show a greater risk of being AO substrates vs. older drugs (based on the growing number of compounds containing aza heterocycle structures, e.g., kinase and GPCR inhibitors).
- Current *in vitro* models – hepatocytes and tissue fractions tend to under-predict *in vivo* clearance.
- No reports of significant AO related DDI to date, but the potential is there as more AO dependent drugs are put on the market (victim drugs).

Esterases

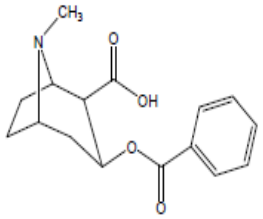
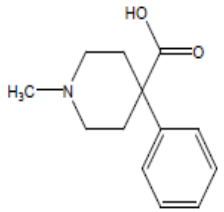
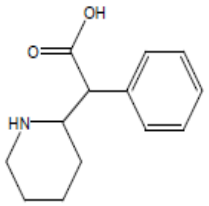
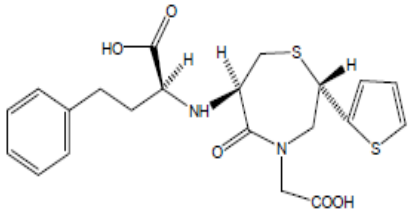
Carboxylesterases (CES): Major Non-CYP Metabolic Enzymes

- Human Carboxylesterases (CESs) are members of the serine hydrolase superfamily
- CESs are categorized as Phase I enzyme that can hydrolyze a variety of ester-containing drugs or pro-drugs (increase bioavailability)
  - Angiotesin-converting enzyme inhibitors (e.g., temocapril, cilazapri)
  - Narcotics (cocaine, heroin)
- In human, carboxylesterases identified for drug hydrolysis belong to the CES1 and CES2 family; CES3, 4A and 5A also in human tissue, but not involved in DM.
- CESs exist on luminal side of ER in microsomes, but also found in cytosol
- CES1
  - Major liver form, also in lung, very low in gut.
  - Monomer MW: 60KDa, exists as a trimer (MW: 180 KDa)
- CES2
  - Major intestinal form, lower in liver, also in kidney and brain
  - Exists as a monomer (MW: 60KDa)

Other Hydrolytic Enzymes Involved in Drug Metabolism

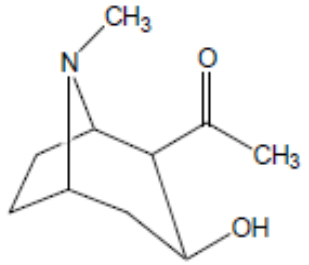
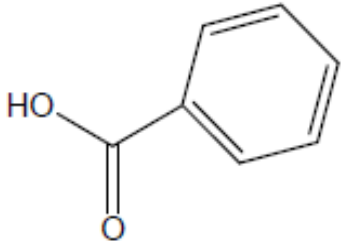
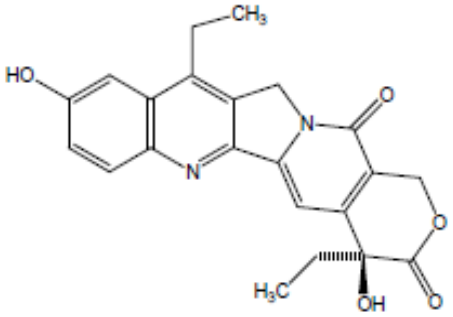
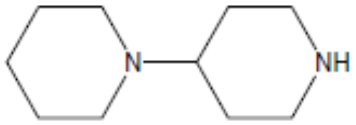
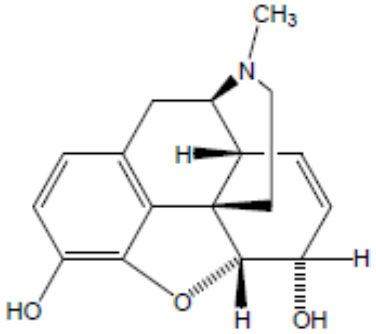
- BChE (Butyrylcholinesterase)
  - Liver (not active?), plasma (secreted from liver)
  - Rough ER
  - Cocaine, CPT-11, aspirin, heroin
- Paraoxonases (Lactonases)
  - PON1, PON2, PON3
  - Liver, plasma
  - Calcium dependent
  - Lovastatin, simvastatin, pilocarpine, prulfiloxacin, organophosphate pesticides
- AADAC (arylacetamide deacetylase)
  - Microsomal (luminal side)
  - Liver, intestine
  - Flutamide, rifampicin, phanacetin
- CMBL (carboxymethylenebutenolidase)
  - Cytosol
  - Liver, intestine
  - Olmesartan medoxomil, faropenem

CES1 Substrate Specificity
(Hosokawa, Molecules, 2008)

Substrate	Alcohol Substituent	Acyl Substituent	Substrate Specficity
Cocaine (methyl ester)	CH_3OH		CES1
Meperidine	CH_3CH_2OH		CES1
Methylphenidate	CH_3OH		CES1
Temocapril	C_2H_5OH		CES1

- CES1 prefers substrates with small alcohol leaving group and large acyl group.
- CES2 prefers substrates with large alcohol leaving group and small acyl group.

CES2 Substrates
(Hosokawa, Molecules, 2008)

	Alcohol Substituent	**Acyl Substituent**	
Cocaine (benzoyl ester)			CES2>>CES1
CPT-11			CES2>>CES1
Heroin		CH_3COOH	CES2>>CES1

Human Esterase Inhibitors and Probe Substrates

- **Non-specific CES inhibitor**
  - Bis(4-nitrophenyl) phosphate (BNPP)
- **CES1 inhibitors**
  - Clopidogrel
  - Nordihydroguaiaretic acid (NDGA)
  - **Digitonin** (Shimizu, DMD, 2014)
- **CES2 inhibitors**
  - Loperamide
  - **Telmisartin** (Shimizu, 2014)
- **AADAC:** Vinblastine potent inhibitor for AADAC and CES2, but not CES1 (Shimizu)

- **CES1 substrate**
  - Trandolapril
- **CES2 substrates**
  - Irrinotecan
  - Fluorescein Diacetate

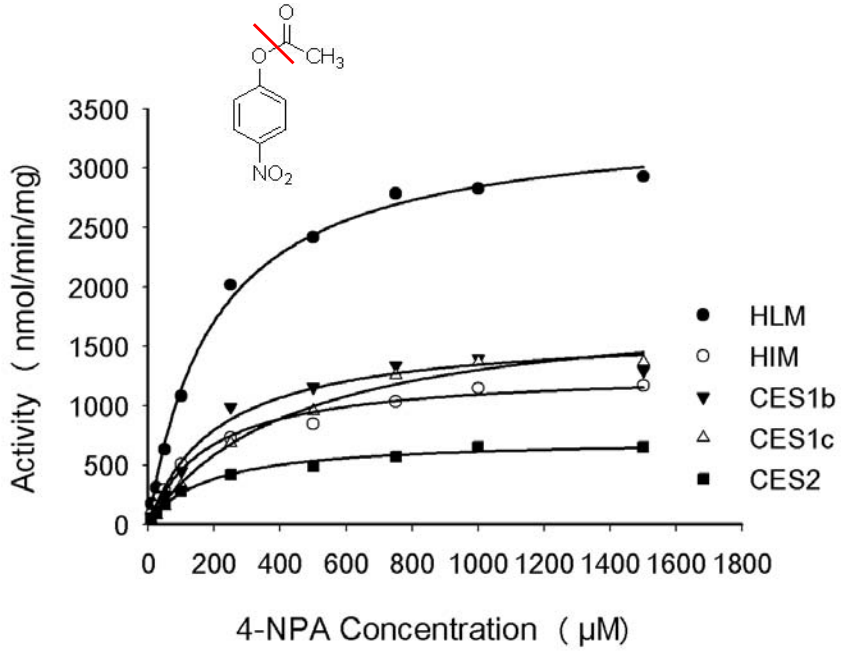
Human CES1 Genes

- Three CES1 gene sequences have been identified in human liver (each has unique accession number in NCBI)
  - CES1a (CES1A1)
  - CES1b: Relative to 1a, 1b lacks Ala18 near N-terminus, CES1a\1b dominant form(s) in liver
  - CES1c: Lacks Ala18 near N-terminus; lacks Gln362 in the proposed active site, several aa near N-terminus are different.

- Recombinant human CES1b, CES1c, and CES2 (Corning® Supersomes™ enzymes: baculovirus-infected High Five insect cells).

Recombinant CESs Show Consistent Characteristics as Human Tissues

	4-NPA as substrate	
Enzyme	K_m(μM)	V_{max}(μmol/mg/min)
HLM	198 ± 17	3.41 ± 0.08
CES1b	208 ± 41	1.62 ± 0.09
CES1c	441 ± 67	1.87 ± 0.11
HIM	182 ± 25	1.29 ± 0.05
CES2	173 ± 22	0.718 ± 0.024

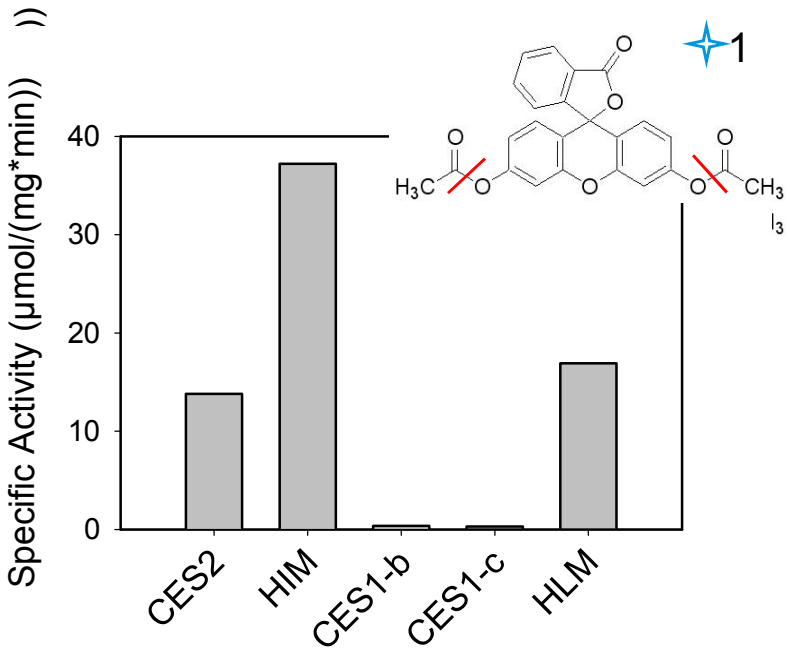


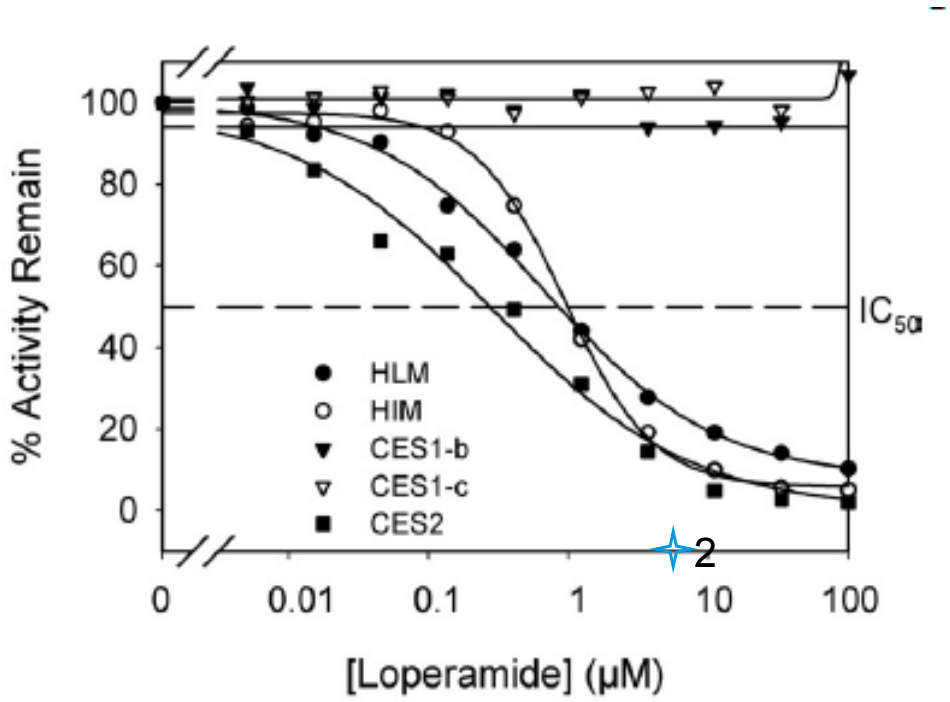
Corning® Supersomes™ CESs:

- CES1b is predominant form in liver for the hydrolysis 4-NPA (K_m matches HLM)
- CES1c is found in liver, higher K_m value due to one mutation in the active site
- CES2 is the predominant form in the intestine (K_m matches HIM)

- CES1b and CES2 activity was found to be similar to human liver microsomes and intestinal microsomes, respectively.

Fluorescein Diacetate is a Probe Substrate for CES2





- Activity for Corning® Supersomes™ CES2 is about 50-fold higher vs. CES1b or 1c (k_{cat} values of CES2 is roughly 100-fold higher than CES1b or 1c based on estimated expression level).
- CES2 is present in both liver and intestine, while CES1 is liver specific – FD hydrolysis in HLM is due to CES2.
- Loperamide, a known CES2-specific inhibitor, inhibits HLM, HIM, and CES2 with similar IC_{50}, while showing no inhibition towards CES1b/c.

Wang J., Williams ET, Bourgea J, Wong YN, and Patten CJ (2011). DMD 39:1329-1333.

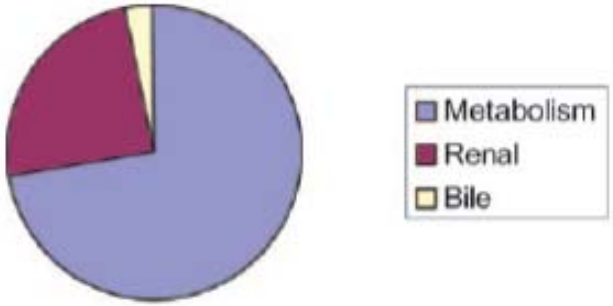
1 50 µM FD was used as substrate
2 5 µM FD was used as substrate

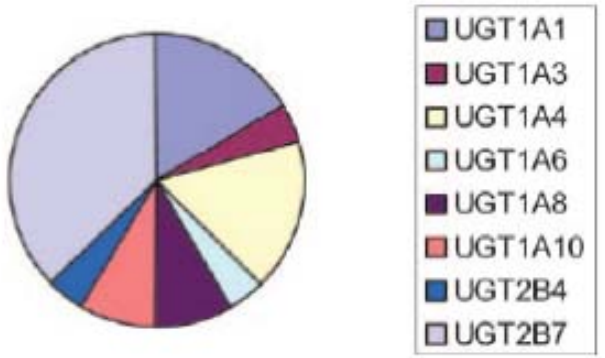
Conclusions: Esterase Enzymes

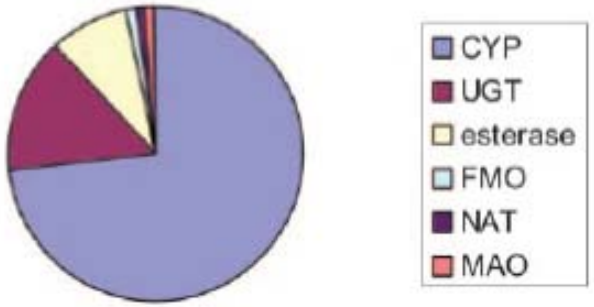
- No reports of clinical DDI involving esterases
- Many potent inhibitors identified, so DDI potential exists
- Alcohol and grapefruit juice inhibit CES (kaempferol, quercetin, not bergamottin)
- Several reported SNPs that reduce activity and cause changes in drug exposure
- *In vitro* tools (e.g., recombinant enzymes, inhibitors) lacking for esterase enzymes other than CES (e.g., ADDAC, PON)

UDP-Gluronosyltransferase

Importance of UGTs for Drug Metabolism







Williams et al., DMD, 2004; Kaivosaari, 2010

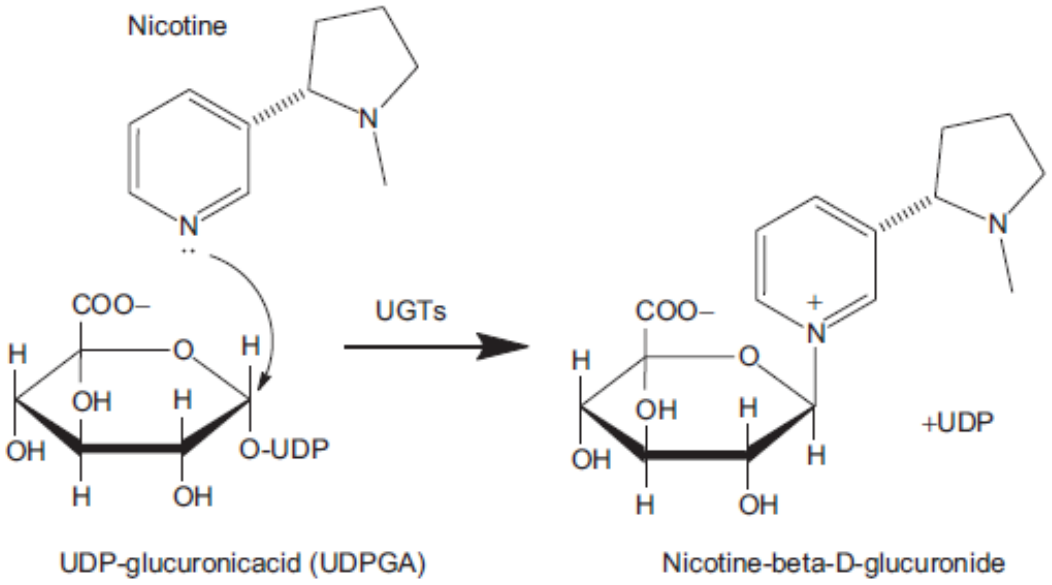
<u>Top 200 prescribed drugs in 2002</u>

- Metabolism is predominant means of elimination
- CYP is major metabolic enzyme, followed by UGTs

- **UGT is listed as major Clearance mechanism for 1:10 drugs**
- **UGT2B7 is major UGT (metabolism of ~40% of drugs), followed by UGT1A1, UGT1A4, and UGT1A9 (combined they account for 47%)**

UDP-Glucuronosyltransferases (UGTs)

- UGTs conjugate glucuronic acid to lipophilic substrates to more water-soluble metabolites, glucuronides, to facilitate excretion
- Glucuronidation reactions include:
  - O-glucuronidation
  - N-glucuronidation
  - Acyl - glucuronidation



UGT Subfamilies

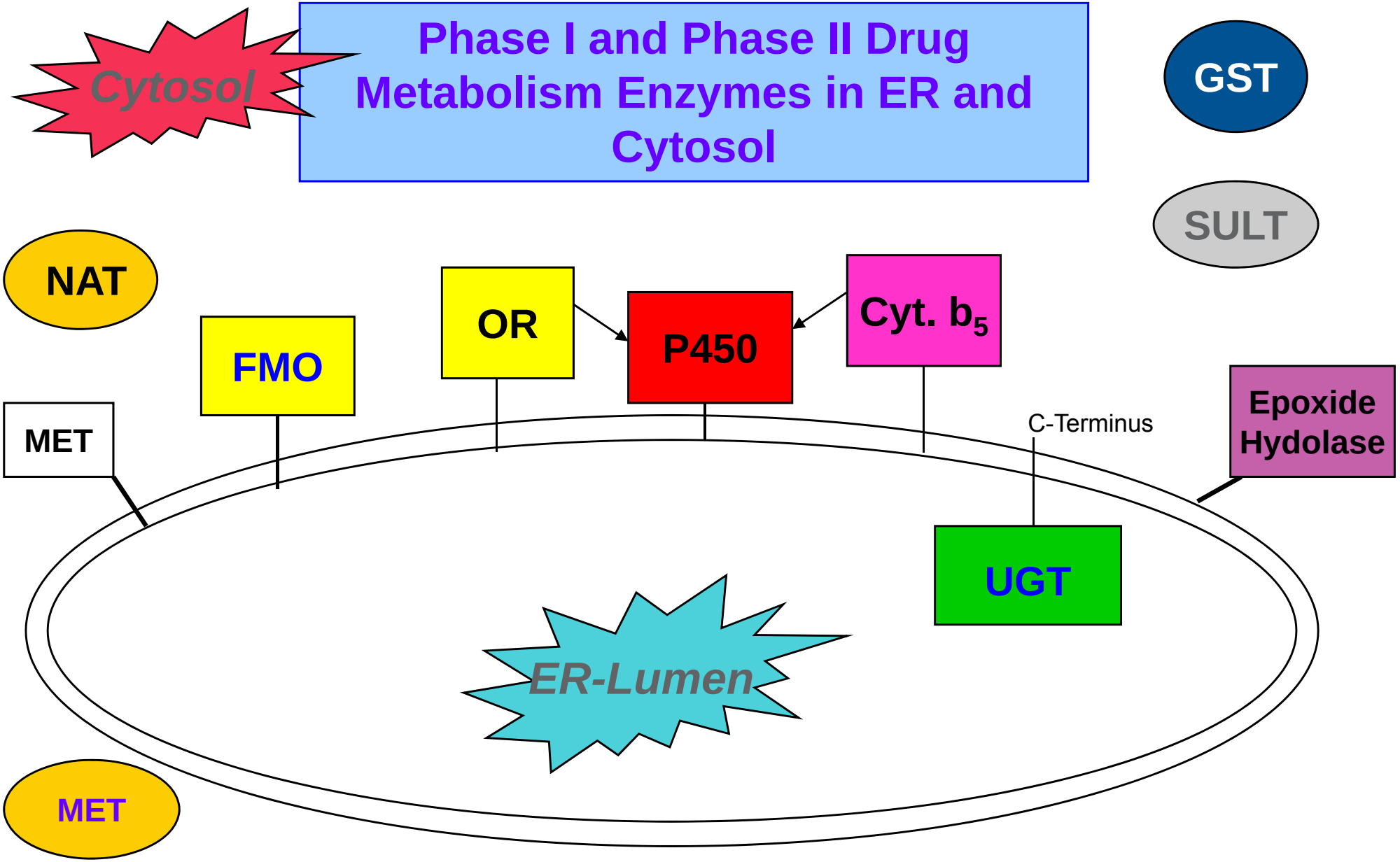
UGT Subfamily:

1A Subfamily (9)	2B Subfamily (7)	2A Subfamily (3)
1A1, 1A3, 1A4, 1A5, 1A6	2B4, 2B7, 2B10, 2B11,	2A1, 2A2, 2A3
1A7, 1A8, 1A9, 1A10	2B15, 2B17, 2B28	

Tissue Expression:

11 UGTs are abundantly expressed in the liver (shown in red), UGTs 1A7, 1A8, and 1A10 are found in GI tract.

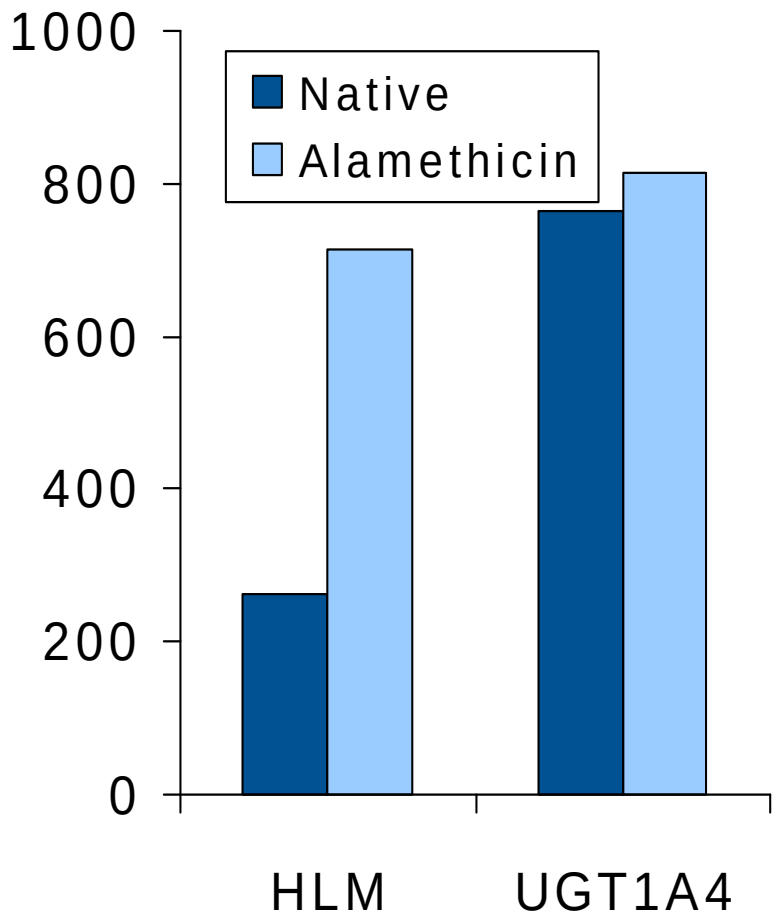
Locations of Metabolic Enzymes



UGT Latency in HLM

- UGTs are located on the lumenal face of the microsome.
- This limits access of substrates and UDPGA and reduces activity (latency).
- Treatment with detergents or pore forming agents reduces latency.
- Treatments can be “tricky” and kill CYPs.
- Preferred activating agent is “alamethicin”.
  - Effective over a broader concentration range
  - Does not inhibit CYPs

➢ Latency or competing long chain fatty acids (LC-FA) may cause under prediction of Cl_{int}
  - Gill, et al., DMD, 2012



Albumin Effect on UGT Activity

- BSA or HAS-FAF removes fatty acids derived from liver/cell homogenization, which competitively inhibit e.g., UGT2B7 and 1A9, i.e., higher K_m in absence of BSA relative to hepatocytes (Rowland, DMD, 2008)
- Typical BSA concentration is 1% to keep non-specific binding at minimum
- Can also use Intestinal Fatty Acid Binding Protein (Rowland, DMD, 2009).
- BSA can, depending on the UGT, impact both the K_m and V_{max}
  - UGTs K_m effected: **1A7**, 1A8, 1A9, **1A10**, 2B4, 2B7, 2B15
  - V_{max} effected: 1A1, 1A6, **1A7**, **1A10**
  - No effect on 2B17 kinetics
  - Effect on V_{max} substrate dependent
  - Similar effect observed with recombinant systems
- Adding BSA to incubations has been shown to improve Cl predictions (Uchaipichat, BJPK, 2006)
- Under prediction also reported using hepatocytes (Naritomi, Y, DMPK Reviews, 2014)
- Improved Cl prediction to within 2-fold by including BSA, alamethicin and both CYP and UGT cofactors (Kilford, DMD, 2009)

UGTs and Drug Interactions

- Fewer relevant drug-drug interactions caused by inhibition of UGTs.
- AUC seldom increase more than 2-fold.

Reasons

- Typically multiple UGTs are involved in metabolism of a single drug.
- K_m values for UGTs are typically high (vs. CYPs), and often higher than the therapeutic plasma levels.
- With exception of UGT1A1, there are relatively few polymorphism that significantly effect enzyme rates.
- UGTs are less susceptible to induction vs. CYPs.

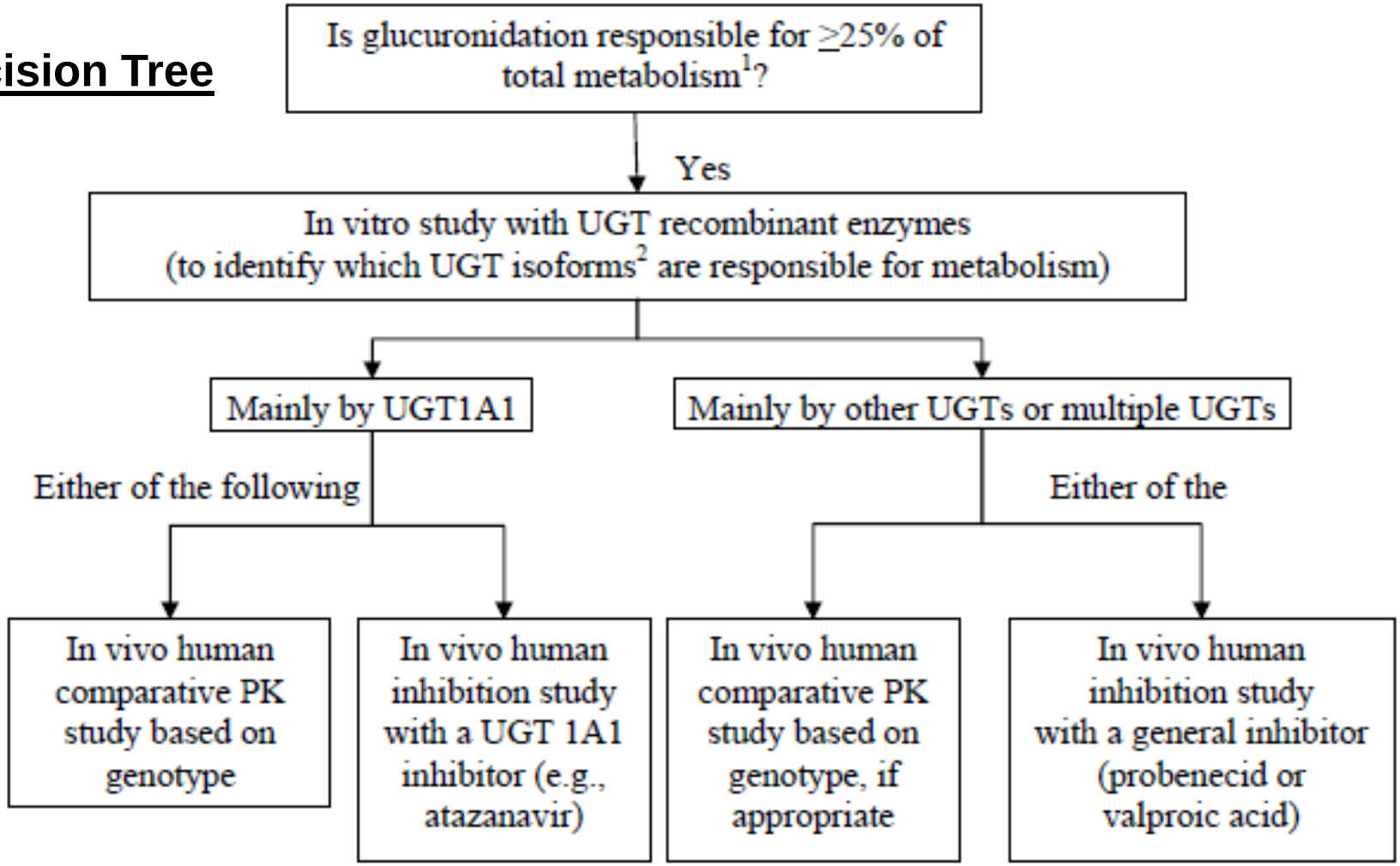
Examples of UGT Drug-Drug Interactions (Williams, DMD, 2004)

Perpetrator Drug	Victim Drug	Effect
Diflunisal	Indomethacin	2-fold increase in AUC
Valproic Acid	Lorazepam	Increase AUC 20%
Valproic Acid	Lamotrigine	Increase AUC 160%
Probenacid	Zomepirac	4-fold increase in AUC
Rifampicin	Mycophenolic Acid	30% Increase in CL

FDA Recommendation for Studying UGTs

"If glucuronidation is a predominant pathway of drug elimination, *in vitro* studies to determine whether the drug is a substrate of UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, or 2B15 are recommended"

UGT Decision Tree



UGT Probe Substrates in Human Microsomes

- UGT1A1: Estradiol (3-glucuronide), Bilirubin
- UGT1A3: 25-Trihydroxy Vitamin D3
- UGT1A4: Trifluoperazine, Amitriptyline/Imipramine (high K_m, 100 µM)
- UGT1A6: Serotonin, 5-hydroxytryptophol (5HTOL), 1-Naphthol
- UGT1A9: Propofol, mycophenolic acid
- UGT1A10: Dopamine
- UGT2B7: AZT, Morphine (6-gluc.)
- UGT2B10: Amitriptyline (low K_m, 10 µM)
- UGT2B15: S-Oxazepam

UGT Selective Inhibitors

- UGT1A1: Bilirubin, Atazanavir (*in vivo*)
- UGT1A4: Hecogenin
- UGT1A6: Naphthol
- UGT1A9: Niflumic acid
- UGT2B7: Fluconazole
- UGT2B10: S-Nicotine

Full panel of chemical inhibitors lacking, inhibitory antibodies do not exist

Kinetic Parameters of Membrane Bound rUGT Enzymes (Corning® Supersomes™ Enzymes)

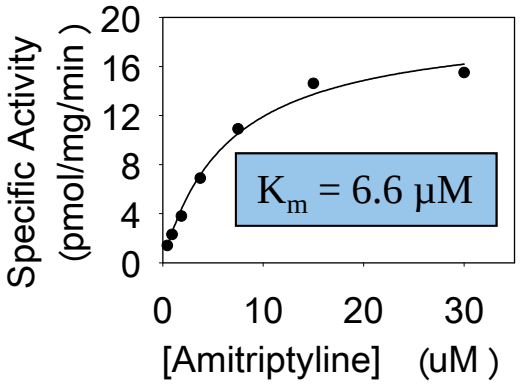
- Recombinant UGTs are available for all major UGTs isoforms (Corning Supersomes enzymes)
- All Supersomes characterized for probe substrate activity (full kinetics)
- Michaelis-Menten constant (K_m) is consistent with value observed in pooled HLM

	Substrate	K_m (µM)	
		Supersomes	**HLM**
UGT1A1	Bilirubin	1.1	0.8
UGT1A4	Trifluoperazine	61	85
UGT1A9	Propofol	10	26
UGT2B7	Morphine (6-Glucuronidation)	766	815
UGT2B10	Amitriptyline	7	10

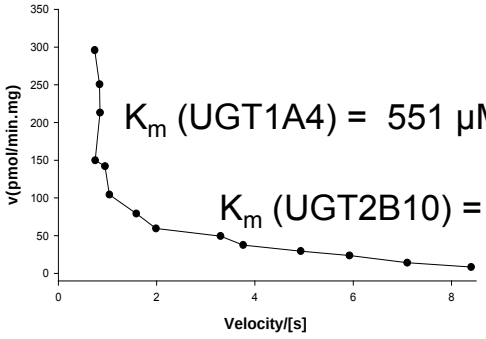
Kinetic Parameters for Amitriptyline and Trifluoroperazine Glucuronidation by UGT2B10 and 1A4 Corning® Supersomes™ Enzymes and HLM

Zhou, DMD, 2010

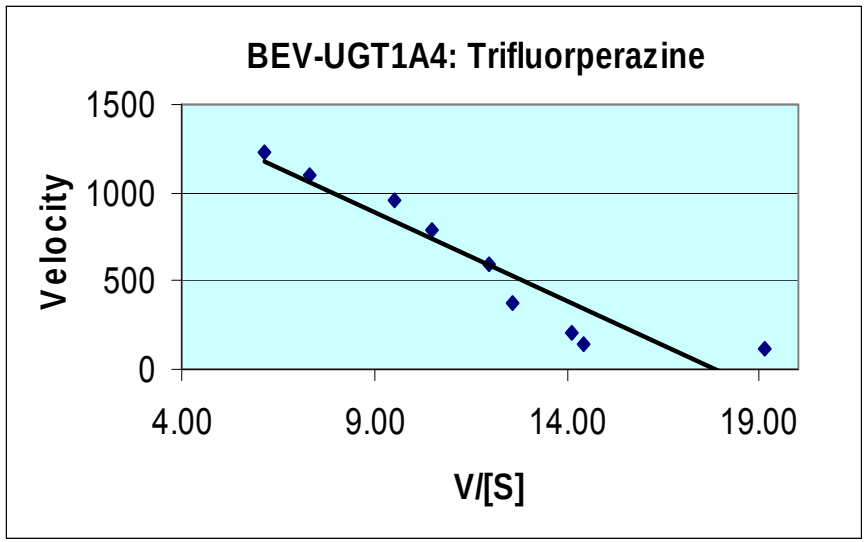
UGT2B10

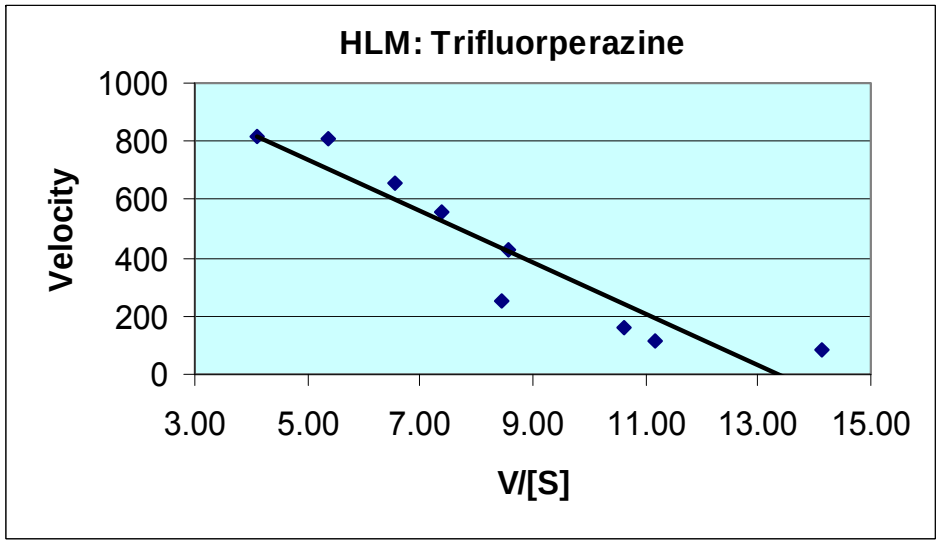


HLM with Amitriptyline (Eadie-Hofstee Plot)



Wang and Patten, 2010





- K_m for TFP 100 µM in both HLM and 1A4 Supersomes enzymes

UGT2B10 Importance in Drug Glucuronidation

- UGT2B10 is the high affinity (low K_m) enzyme for Amitriptyline N-glucuronidation (Zhou, DMD, 2010).
- UGT2B10 glucuronidates tertiary amines (not active for primary or secondary amines).
- High affinity UGT enzyme for amitriptyline, imipramine, and diphenhydramine tertiary amine substrates – important for Clearance at low doses administered.
- Recently shown to be involved in Desloratadine (Clarinex®) metabolite formation (Kazmi, DMD, 2015).
- Splice-site mutation (non-functional protein) detected in African and Asian populations, 45% and 8%, respectively, 1% in Caucasians (Fowler, JPET, 2015)
    - 100-fold lower intrinsic clearance of RO5263397 in hepatocytes homozygous for the splice-site variant allele.

Conclusions: UGTs

- *In vitro* tools for studying UGTs still lacking compared to CYPs.
- *In vitro* models (HLM, hepatocytes) tend to under predict Cl_{int} for drug glucuronidation pathways.
  - Predictions with HLM can be improved by adding BSA, alamethicin and CYP/UGT cofactors to incubations.
- UGT2B10 shown to be important for tertiary amine drugs.
- UGT2B10 polymorphism recently identified (splice variant, no activity), with high frequency in African and Asian populations.

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