



Strategies for In Vitro Metabolic Stability Testing

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Presentation Overview

- Overview of in vitro metabolic stability testing
- In vitro model systems and assay conditions
- In vitro / in vivo Extrapolations
(predicting in vivo clearance)



The Ideal Drug

ADME/Drug Development View

- **Administration is oral and the drug is easily absorbed**
 - Good permeability and aqueous solubility (class I compounds)
- **Metabolically stable (but not too stable)**
 - Swallow the pill once a day
- **Predictable Metabolism**
 - Linear drug metabolism kinetics
- **Balanced Clearance**
 - Renal or biliary secretion of parent drug
 - Metabolism to limited number of inactive products
 - Metabolism by several P450s (>2)
 - Metabolism should not depend on Polymorphic P450s
- **Not an Inhibitor or Inducer of ADME Enzymes**
 - P450, UGT, and MDR1 (P-gp)
- **Small First-Pass Effect (liver or gut)**
- **Wide Therapeutic Index**



Advantages of Enhancing Metabolic Stability

- Increase bioavailability and half-life; less frequent dosing
 - Improved patient compliance
- Better congruence between dose and plasma concentration
 - Reduce need for therapeutic monitoring (expensive)
- Reduction in turnover rates from different pre-clinical species
 - May improve extrapolations from animal data to humans
- Lower patient to patient variability in drug levels
 - Patient variability largely due to differences in drug metabolism capacity
- Reduce the number and significance of active metabolites
 - Reduces the need for further studies of metabolites in animals and humans

Ariens, et al., Strategies in Drug Research (1982)



Timing of Metabolic Stability Testing

- Early Discovery
 - HTS screens for loss of parent
 - Determine in vitro $T_{1/2}$
 - Rank order compounds, SAR studies
 - Generally test in liver microsomes from rat and human (pools)
- Development
 - Determine Cl_{int} with full Kinetics (V_{max} and K_m)
 - Use scaling factors and liver flow models to predict in vivo hepatic clearance



Species Differences

- Variations in primary sequence of CYP between species can result in differences in substrate specificity/activity
- Levels of CYP isoforms may differ between species
- Interspecies differences in enzyme inhibition
- Cannot make cross-species predictions regarding metabolic stability

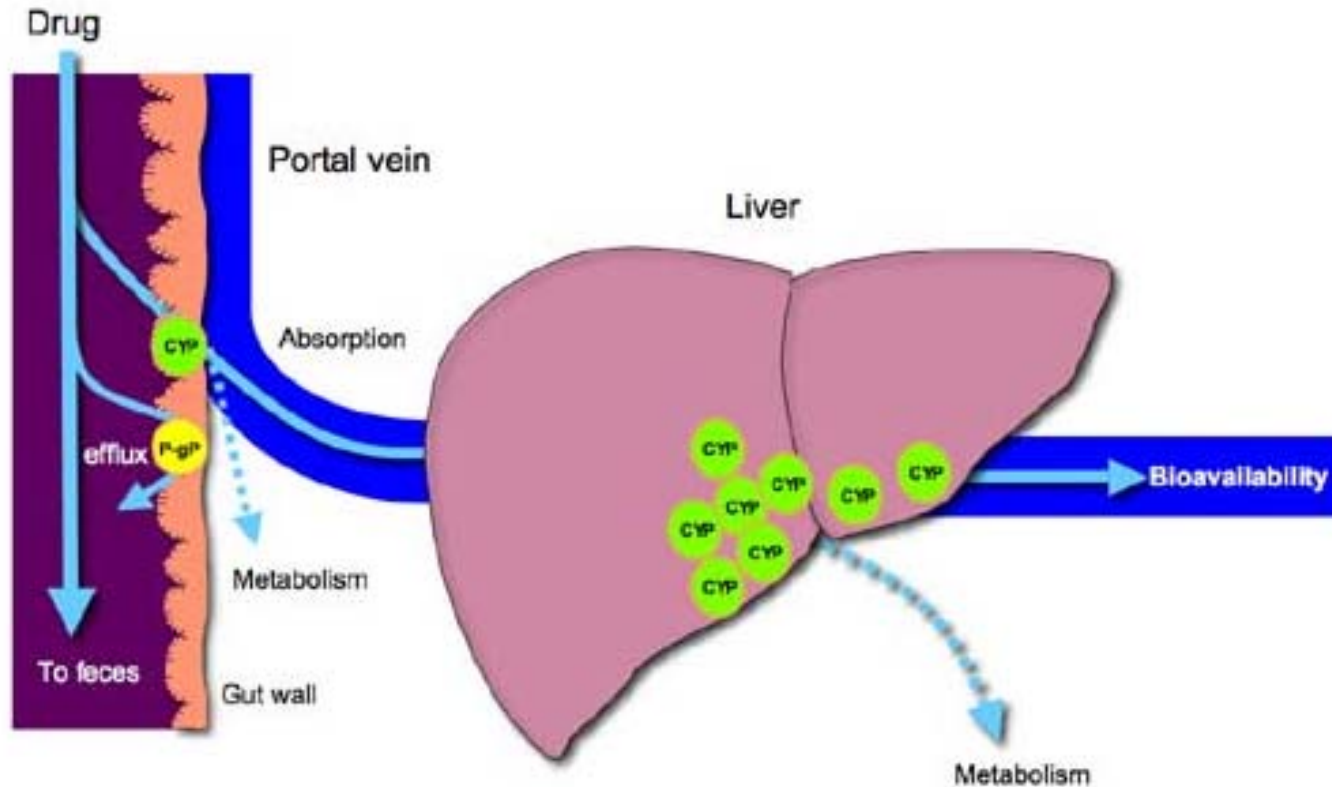


Sites of Drug Metabolism

- Primary Site of Metabolism is Liver
 - Extra-Hepatic metabolism can be important
 - “Portals of entry” tissues often have significant drug metabolism capability
 - GI (small intestine) is major site of metabolism next to liver
 - Other sites include: lung, skin, nasal mucosa, kidney
- First Pass Effect
 - Drug absorbed in small intestine and transported to liver via portal vein
 - Extensive metabolism in liver and/or intestine
 - Limited systemic availability



Bioavailability for Oral Medication (F)



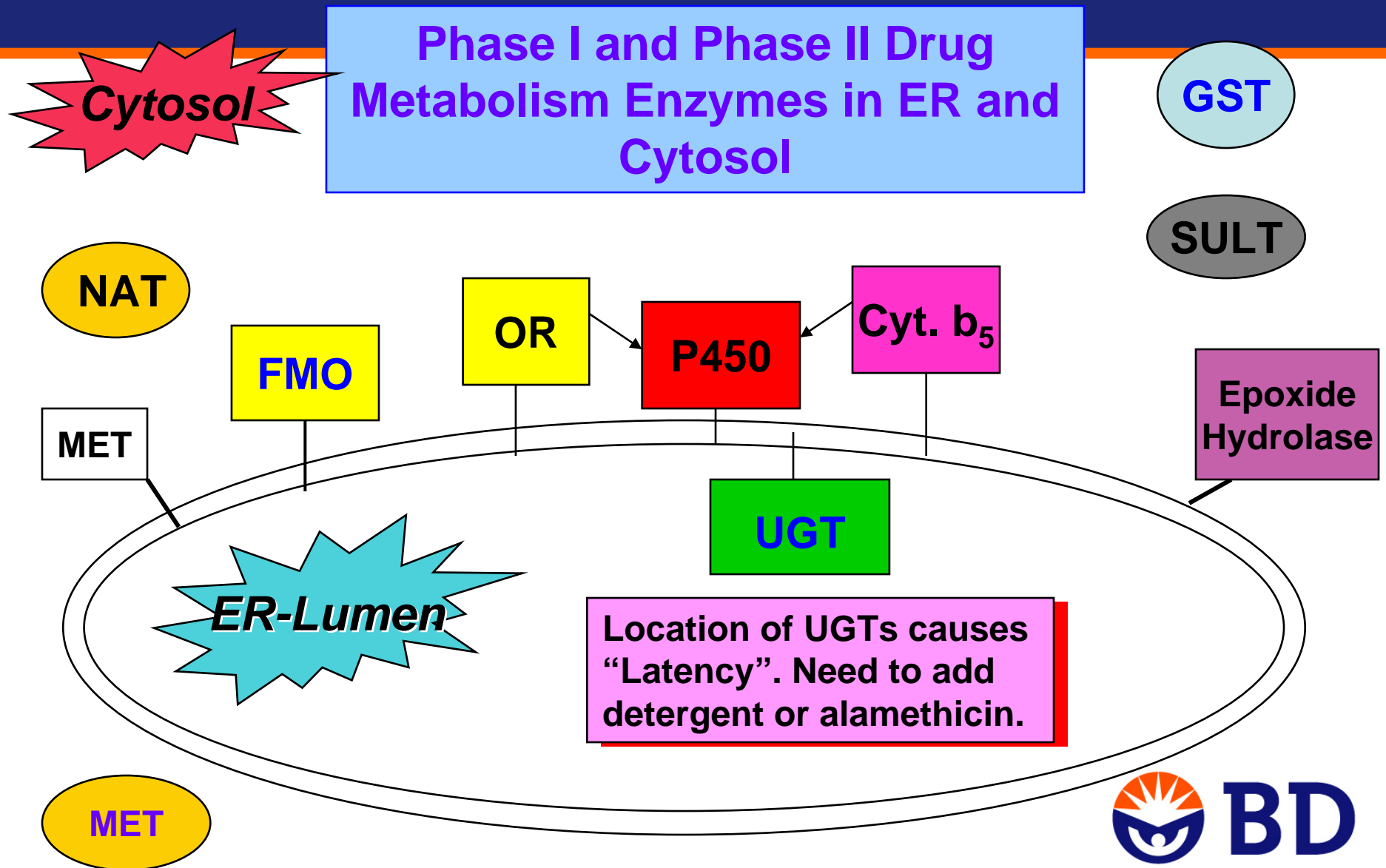
Phase I and Phase II Biotransformation

Metabolism/Biotransformation is divided into two groups

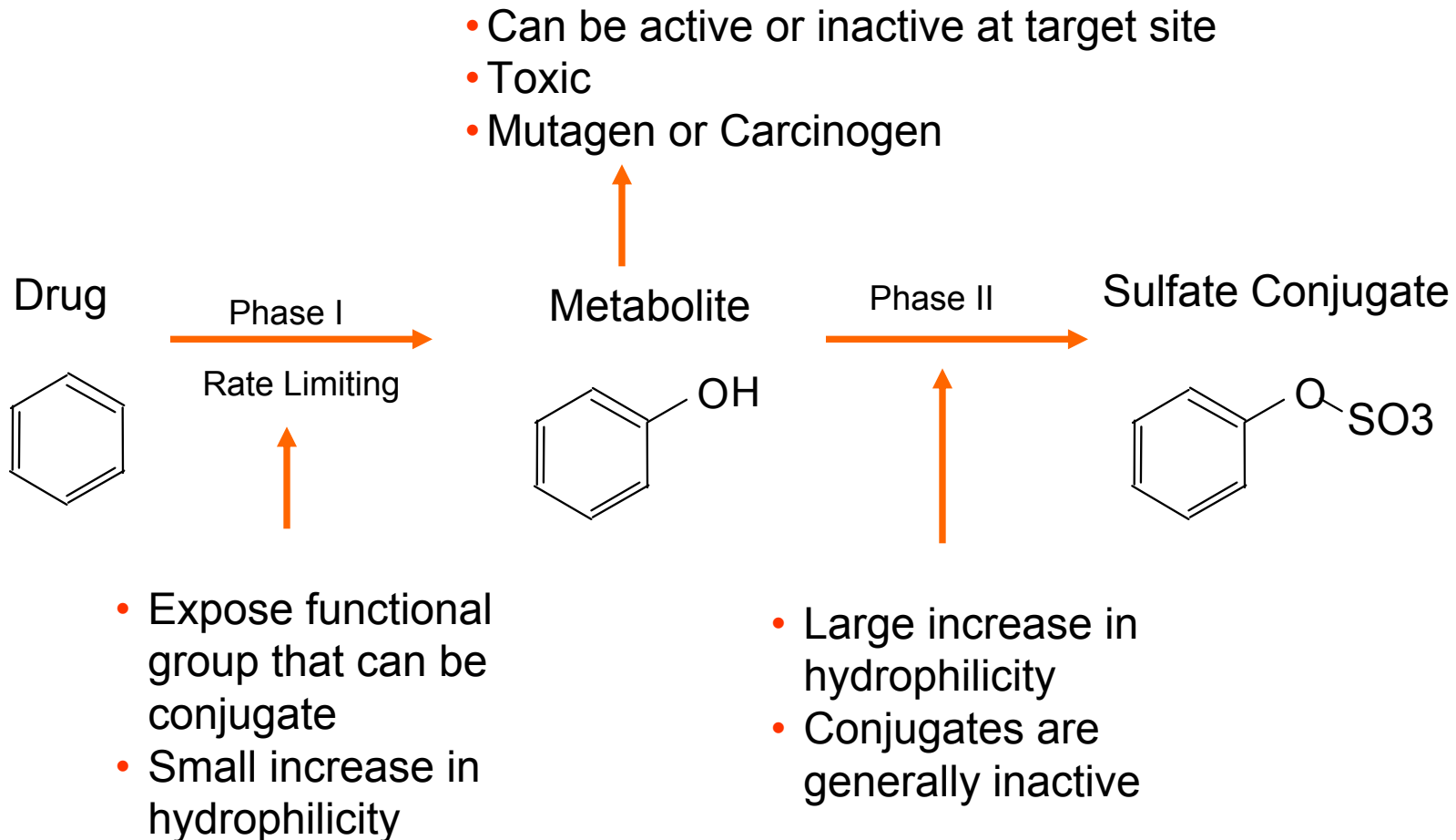
- **Phase 1:** Addition or unmasking of functional, polar moiety
 - Oxidation (P450 or FMO)
 - Hydrolysis (Esterases)
 - Most typical is Hydroxyl group created or exposed (de-alkylations)
- **Phase 2:** Conjugation with small, endogenous substance, often takes advantage of functional group added in Phase I
 - UGT is most important Phase 2 enzyme (conjugates with GA)
- **End Result (Phase 1 + 2):** Increase polarity and aqueous solubility of drug which facilitates elimination from the body



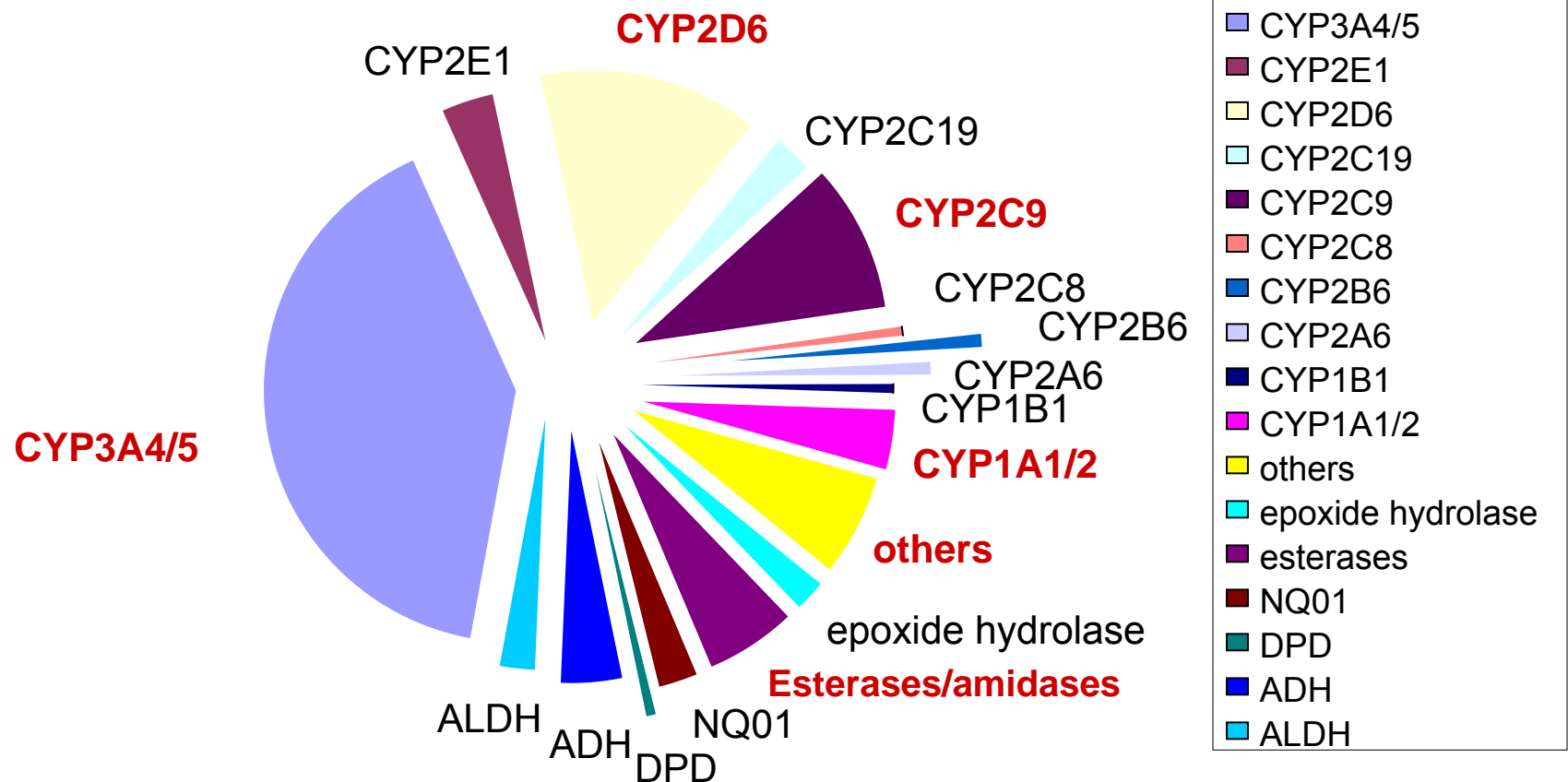
Location of Metabolic Enzymes



Phase I and Phase II Metabolism

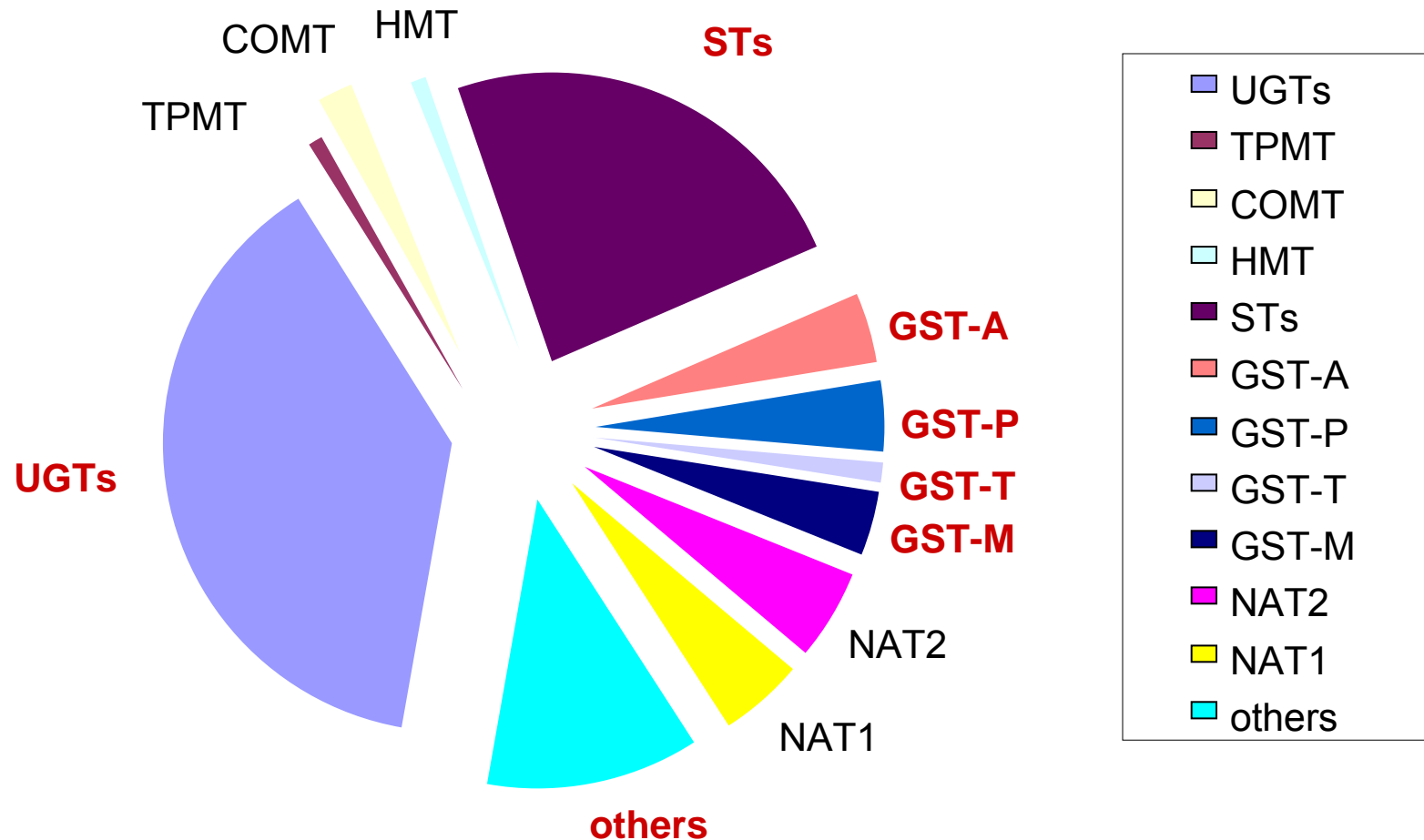


Human Phase I Enzymes of Drug Metabolism



CYP: cytochrome P450, NQ01: NADPH:quinone oxidoreductase (DT diaphorase);
 DPD: dihydropyrimidine dehydrogenase; ADH: alcohol dehydrogenase; ALDH:
 aldehyde dehydrogenase

Human Phase II Enzymes of Drug Metabolism



HMT: histamine methyltransferase; TPMT: thiopurine methyltransferase;
COMT: catechol O-methyltransferase; UGT: Uridine Glucuronosyl-S-Transferases;
ST: Sulfotransferase; GST: Glutathione-S-Transferases

Model Systems for Predicting Drug Clearance In Vivo

Whole Animal

Whole Organ (liver)

Organ Slices

Cell lines (HepaRG) **Hepatocytes**

Microsomes/S9

Expressed Enzymes

Purified Enzymes

← Used for Metabolic Stability Testing



In Vitro Systems

I. Hepatocytes: Prepared from fresh human livers (organ donors)

- Gold-Standard for DM Studies
 - Contain all the enzymes/transporters and co-factors for drug metabolism
 - Metabolic stability (Screening for long half-life drugs)
 - Metabolite profiling (structures of metabolites)
 - Liver toxicity studies
 - Enzyme induction studies (P450 induction)
 - *In vitro* / *In vivo* scaling

Not used for:

- Enzyme Mapping/Reaction Phenotyping
- DDI

II. Liver Slices

- Similar to hepatocytes in that they contain the full complement of hepatic DMEs
 - Harder to prepare than other systems
 - Seldom used for ADME studies



In Vitro Systems

III. Liver Microsomes

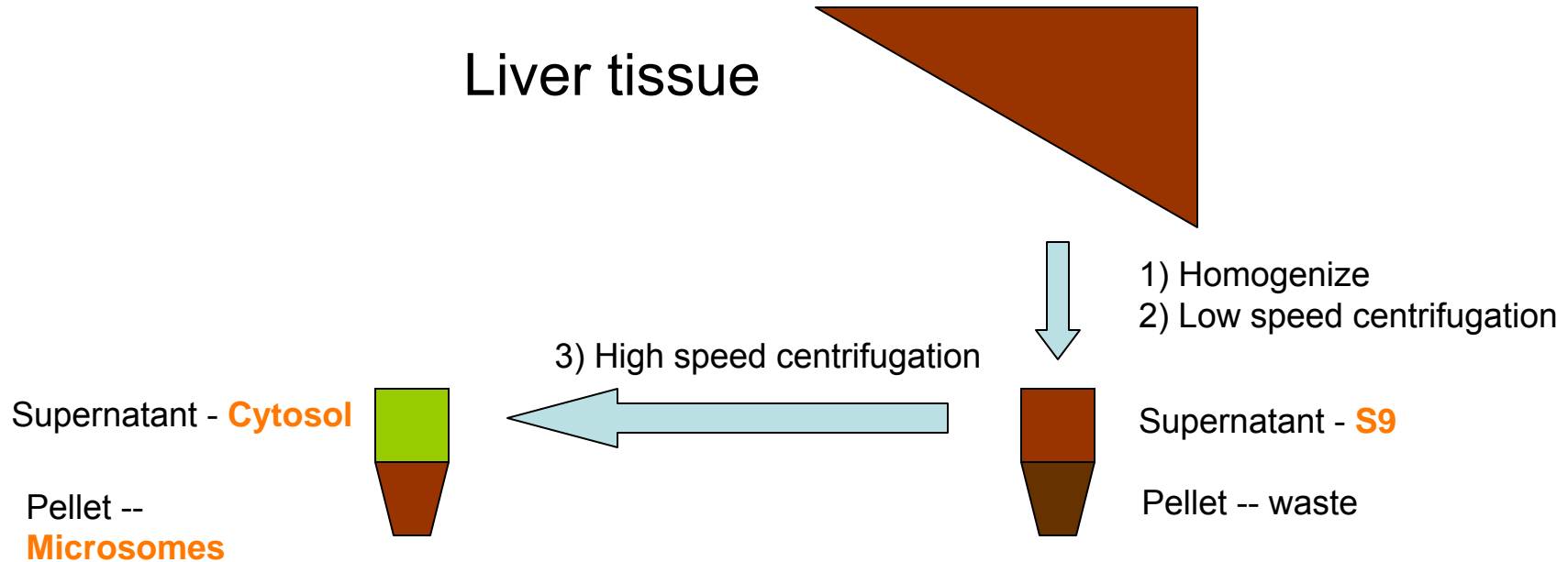
- Contain all P450s, FMOs, and UGTs
- Easy to prepare and can be stored for long periods (-80°C)
- Withstand several Freeze/Thaw cycles
- Can make Donor Pool; 20 to 150 donors (average patient in population)
- Uses: Drug half-life (in vitro scaling), DDI, metabolite profiles, enzyme mapping, mechanistic studies

IV. Liver S9

- Same as microsomes, but contains cytosolic enzymes (SULT, GST, XO, ADHs, NATs)
- Same advantages and uses as HLM
- P450 activity ~five-fold lower vs HLM



Method of Tissue Fraction Manufacture



S9 = Both cytosol and microsomes = Phase I & II enzymes

Cytosol = Soluble proteins (phase II enzymes) = NAT, GST, SULT

Microsomes = membrane proteins (phase I enzymes) = P450, UGT, FMO

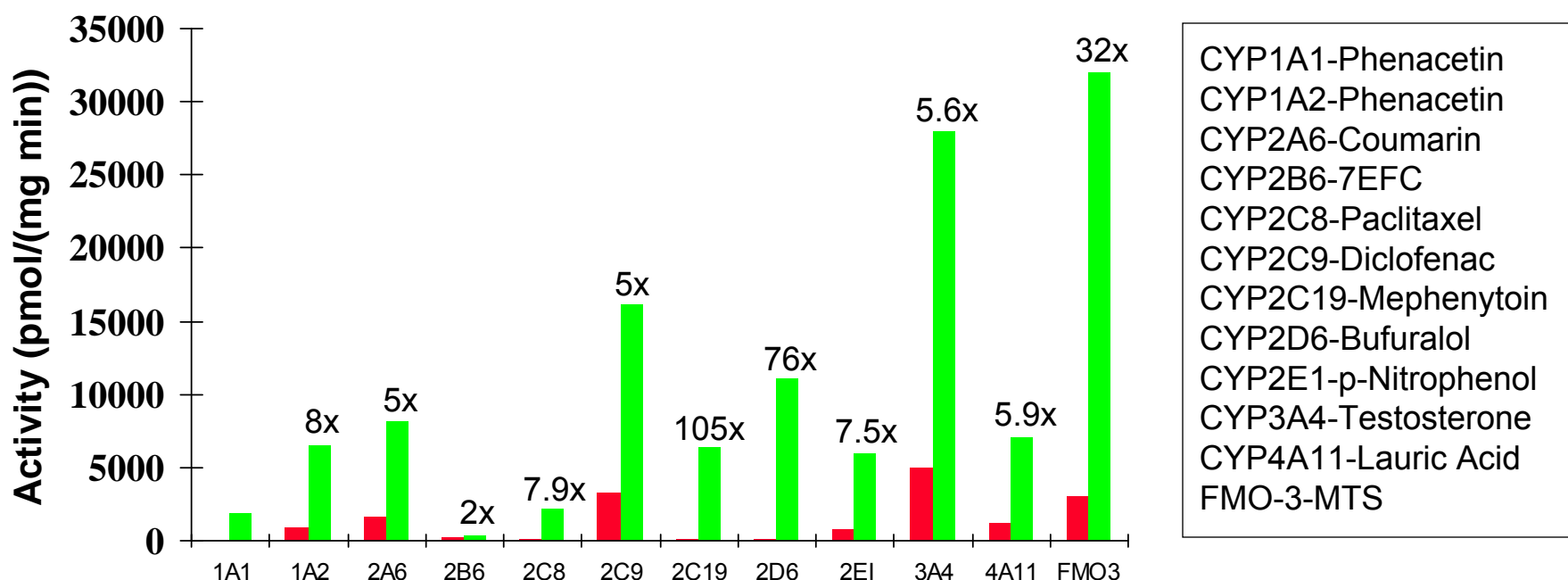
In Vitro Systems

V. Recombinant Enzymes (e.g. BD Supersomes™ Enzymes)

- Single DME expressed in a cell line
 - Baculovirus-insect cells
 - Yeast
 - E. coli
 - Mammalian cells
- Co-expressed with P450 OR and in some cases with b5
- Reaction phenotyping (EM), DDI, mechanistic studies
- Can be useful for studying highly stable compounds (more active than HLM)
- Requires RAF or ISEF for comparing to HLM activity



Activity Comparison (per mg protein) BD Supersomes Enzymes versus HLM



Assay Incubation Conditions

- **Assay buffer**
 - Phosphate or Tris buffers
 - 100 mM KPO₄ (pH 7.4) with MgCl₂ (~3 mM) is most common
- **Co-factors**
 - NADPH or NADPH generating system: can use either, no significant difference (GS contains MgCl₂)
 - Phase 2 co-factors: UDPGA (10 mM), PAPS (1 mM) are saturating
- **BSA or HSA (2%)** (Miners, et al., DMD, 2008)
 - Sequesters unsaturated long chain FFA released from microsomal membranes during the course of an incubation
 - Shown to decrease K_m for UGT2B7, UGT1A9, and CYP2C9 (increase C_{l, int}), and improve IV-IVE predictions (FFA act as competitive inhibitors)
 - Other CYPs and UGTs may be effected
 - HAS need to be higher grade: HAS-FAF grade
 - Albumin binds drugs: need to measure fu of drug in incubation
- **UGT Reactions**
 - Alamethicin (25 ug/mL) to reduce latency (Alamethicin forms pores in microsomal membrane)
 - Saccharic acid 1,4-lactone (5 mM) to inhibit endogenous glucuronidase activity
- **Organic Solvents for Dissolving Test Compounds** (Busby, et al., DMD, 1999)
 - Organic solvents can inhibit P450s
 - Acetonitrile: up to 2%
 - Methanol: up to 1% (caution: MeOH can cause formaldehyde adducts and artifactual loss – Yin, et al, 2001)
 - DMSO: up to 0.2%
 - UGTs can also be inhibited by organic solvents
- **Non-Specific Binding to Microsomal Membranes**
 - Test compounds can bind non-specifically to matrix (microsomes)
 - Extent of binding is compound specific
 - At low protein concentrations (<0.1 mg/ml) binding may be negligible
 - Binding between HLM and rCYP are often considered the same (Stinger, et al., DMD, 2009)
- **Protein/Cell Concentrations**
 - Hepatocytes: 0.25 X 10⁶ cells/0.25 mL (24-well plate)
 - Liver microsomes: 0.5 mg/mL
 - Liver S9: 2.0 mg/mL
 - cDNA-expressed enzymes: P450 activity needs to be scaled to HLM activity (RAF, ISEF), 100 pmol/ml is common



Drug Discovery

HTS Metabolic Stability Assays

- Fully automated HTS Metabolic Stability assays
 - 384-well plate based assays (>1000 compounds/week)
 - Reduction in assay incubation volumes: as low as 15 μ l (~10 μ g HLM per well)
 - Loss of parent method
 - Typically screen with rat and human liver microsomes
 - Decrease the number of samples using sample pooling methods (e.g. cassette analysis, Halladay, et al. DM Letters, 2007)
- Combine with LC/MS for analysis
 - Fast, sensitivity and selective
 - High solvent flow rates, short columns, and column switching minimize LC run times





Predicting Drug Clearance In Vivo from In Vitro Data

Drug Clearance

- Clearance (CL) is a measure of elimination of a drug from the body
- Text book definition: volume of blood cleared of drug per unit time
 - Drug Half Life = $0.693 \times (\text{Volume Distribution} / \text{CL})$
- Determines how often you need to take a drug to achieve the desired (therapeutic) effect
- Clearance Mechanisms
 - CL-met vs. CL-renal vs. CL-biliary vs. CL-other
 - CL is additive:

$$\text{CL (Systemic)} = \text{CL}_{\text{Hepatic}} + \text{CL}_{\text{Renal}} + \text{CL}_{\text{Biliary}} + \text{CL}_{\text{Other}}$$



Assumptions Required for Predictive Value of Microsomes

- Metabolism is a major mechanism of clearance
 - $CL_{\text{met}} \gg CL_{\text{renal}} + CL_{\text{biliary}} + CL_{\text{other}}$
- Liver metabolic rate \gg all other tissues
- Oxidative metabolism predominates
 - P450 + FMO (UGT) \gg all other metabolic reactions
- In vitro enzyme specific activity \sim in vivo enzyme specific activity



Intrinsic Drug Clearance

- CL_{int} : enzyme-mediated clearance that would occur without physiological limitations (e.g. protein binding, hepatic blood flow)
 - Assumes unbound (free) drug concentrations
 - $CL_{int} = V_{max}/K_m$ or $0.693/t_{1/2}$ (half-life)
 - V_{max} = pmol product/min*mg protein
 - K_m (Michaelis-Menten Constant) = μM
 - CL_{int} units = $\mu L/min \cdot mg$ microsomal protein (or # hepatocytes)



Hepatic Scaling Factors for Whole Liver Intrinsic Clearance

- Microsomal SF (MSF)
 - 40 mg microsomal protein/gr liver (Hakooz, 2006)
- Hepatocellularity SF
 - 99 million cells/gr liver (Barter, 2007)
- Liver Weight (LW): 1400 grams
- Body Weight (BW): 70 kg
- $CL_{u,H,int} = (CL_{u,int} \times MSF \times LW) / BW$
- Units = $\mu\text{L}/\text{min} \cdot \text{kg BW}$ (or $\text{mL}/\text{min} \cdot \text{kg BW}$)
- Whole liver CL: $CL_{u,H,int}$ and $CL'_{int,.....}$ both forms are used

$$CL_{u,H,int} = CL_{u,int} (V_{max}/K_m) \times 40\text{mg microsome/gr liver} \times 1400 \text{ gr liver/kg BW}$$

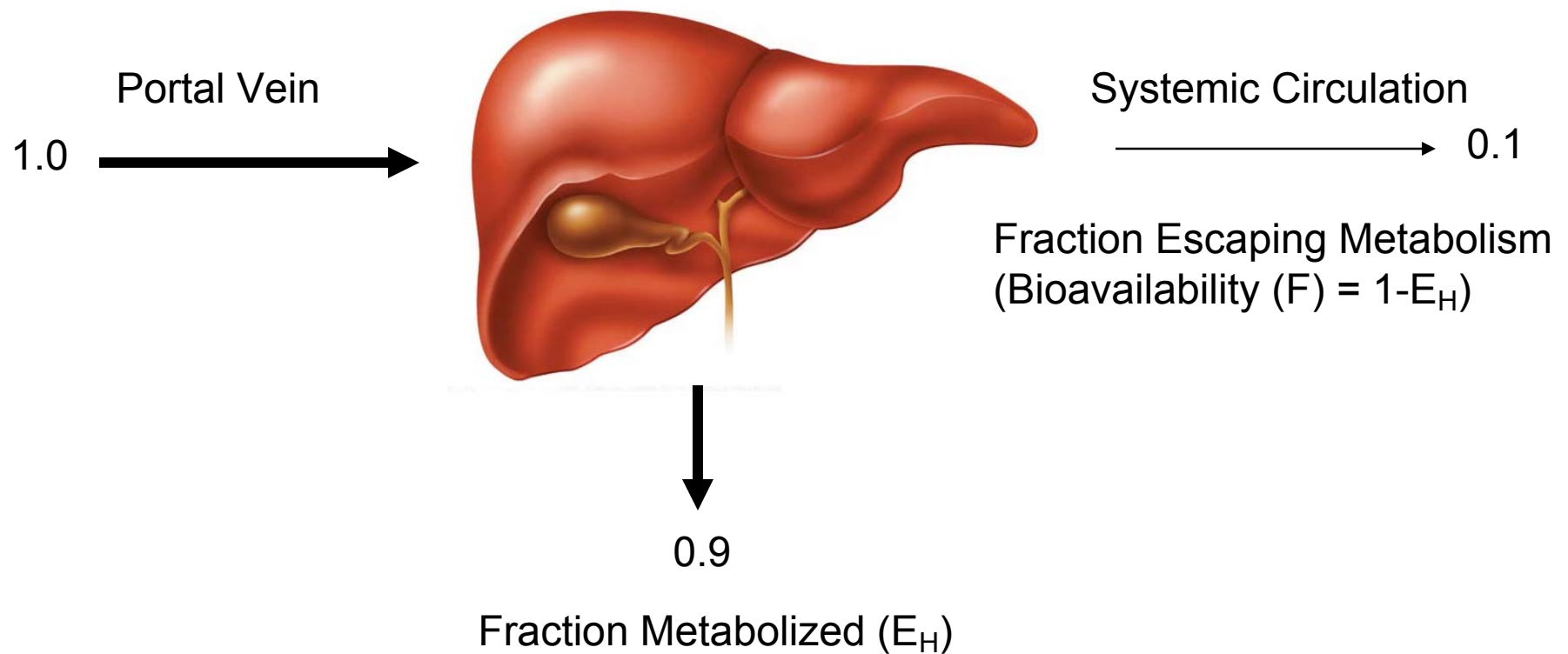


Hepatic (Blood) Clearance (CL_H)

- Organ Clearance (steady state) = $Q \cdot (C_{in} - C_{out}) / C_{in}$
 - Q = Blood Flow
 - C = Drug concentration
- Extraction Ratio (ER) = $(C_{in} - C_{out}) / C_{in}$
- $CL = Q \cdot ER$
- In Vivo Blood Clearance (CL_H) is predicted using a Flow Model such as the Well-Stirred (venous equilibration) Model
 - $CL_H = Q_H \cdot (f_u \cdot CL_{u,H,int}) / (Q_H + f_u \cdot CL_{u,H,int})$,
 - with $ER_H = (f_u \cdot CL_{u,H,int}) / (Q_H + f_u \cdot CL_{u,H,int})$,
 - and $CL_H = Q_H \cdot ER_H$
 - **f_u = Unbound fraction of drug (not bound to protein)**
 - Hepatic Blood Flow = 20.7 ml/min*kg BW (or ~90 L/hour)
- Well-stirred model: assumes homogeneous distribution of drug in liver (mixed well throughout liver). For well-stirred model, the drug concentration coming out of liver equals the intra-cellular drug concentration.
- Only unbound drug crosses cell membrane (unbound drug concentration in plasma and liver water are identical)
- Correcting for non-specific binding to matrix can improve predictions (Riley, et al. DMD, 2005)
 - Measure non-specific binding directly (equilibrium dialysis – e.g. RED device)
 - Estimate from octanol:water partition coefficient (Stringer, et al, DMD, 2009)
 - $CL_H = Q_H \cdot (f_u \cdot (CL_{u,H,int} / f_{u,inc})) / (Q_H + f_u \cdot (CL_{u,H,int} / f_{u,inc}))$



Hepatic Extraction Ratio



$$CL_H = Q_H \times E_H = 90 \text{ L/hour} \times 0.9 = 81 \text{ L/hour}$$



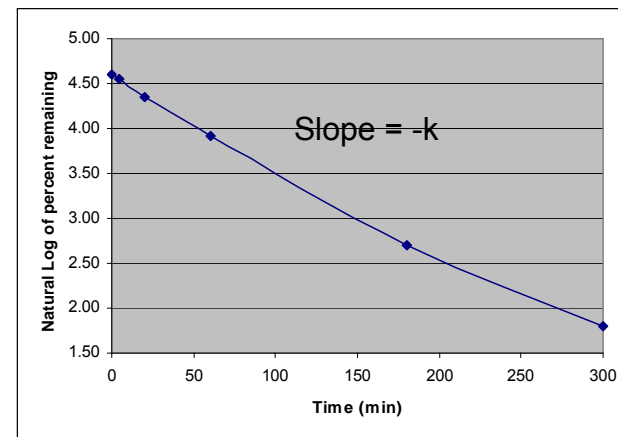
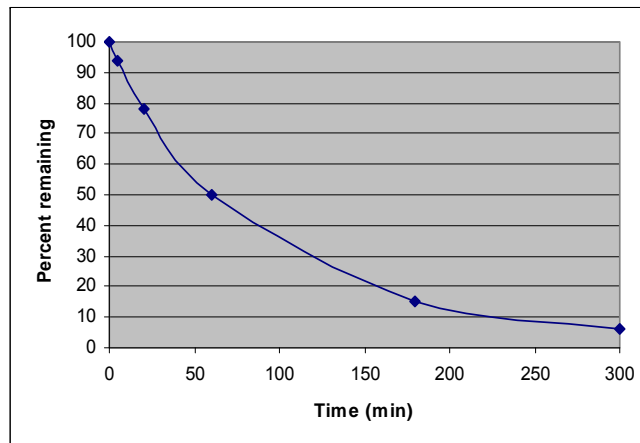
Flow Limited and Capacity Limited CL_H

- Flow Limited (*high CL drugs*)
 - $f_u * CL_{u,H,int} \gg Q_H$
 - $CL_H = Q_H * (f_u * CL_{u,H,int}) / (f_u * CL_{u,H,int}) = Q_H$
 - E.g. propranolol, lidocaine, morphine
- Capacity Limited (*low CL drugs*)
 - $f_u * CL_{u,H,int} \ll Q_H$
 - $CL_H = Q_H * (f_u * CL_{u,H,int}) / Q_H = f_u * CL_{u,H,int}$
 - E.g. warfarin, phenytoin, quinidine, tolbutamide
 - Tolbutamide CL increases in hepatitis patients
 - Due to increase in f_u (no change in Cl_{int})



Drug Half-Life Determination

Time (X)	Percent (Y)	LN%	
0	100	4.61	
5	94	4.54	
20	78	4.36	
60	50	3.91	
180	15	2.71	
300	6	1.79	
Slope = - k =		-0.0115	
t(1/2) = 0.693/- k =		60.2	min



- Loss of parent method used most frequently in Discovery Stage (HTS assays)
- Measure disappearance of low concentration of drug (<5 or 10 μM)
- When $S \ll K_m$ $T_{1/2} = \ln 2 / -k$ (or $0.693 / -k$)
- Terminal elimination rate constant (-k): slope of linear regression from natural log percentage substrate remaining versus incubation time
- Intrinsic clearance (CL_{int}) in units of mL/min/kg
- $T_{1/2}$ units = min - need to include mL/mg into CL_{int} calculation to convert to mL/min*mg)

$$CL_{uH,int} = 0.693 / \text{in vitro } T_{1/2} \times \text{mL incubation/mg protein} \times 40\text{mg microsome/gr liver} \times 1400 \text{ gr liver/kg BW}$$



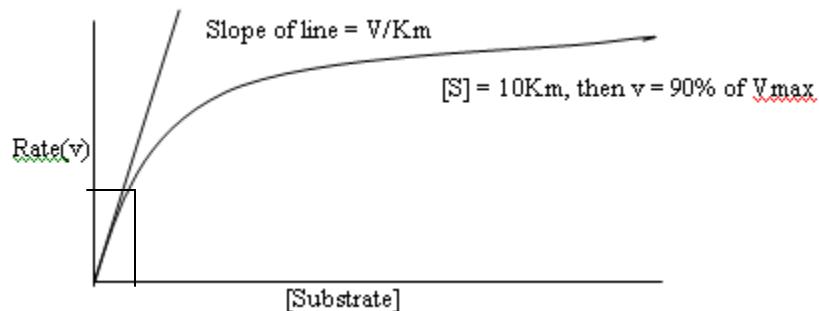
Substrate Concentration Plots

Michaelis-Menten Kinetics

$$v = \frac{V_{\max} * S}{K_m + S}$$

Rectangular Hyperbola

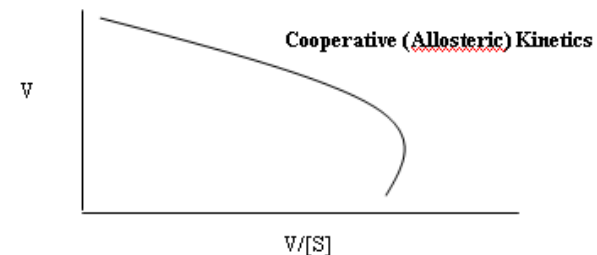
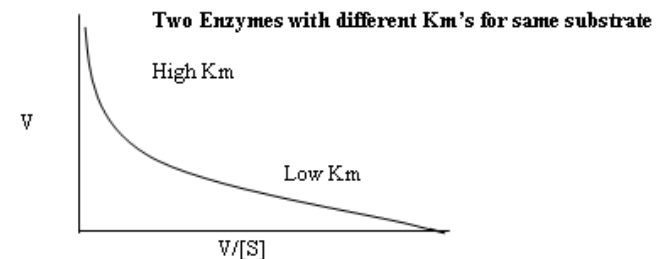
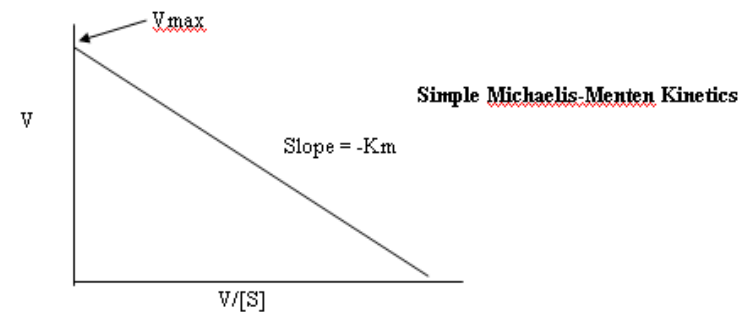
Direct Plot: V vs $[S]$ $V = V_{\max}$



K_m (substrate concentration that gives $\frac{1}{2} V_{\max}$)

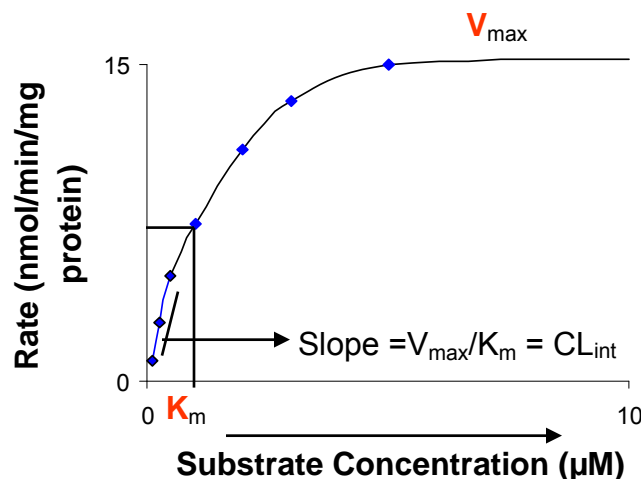
Linear Transformation

Eadie-Hofstee Plot: $v = V_{\max} - K_m (v/[S])$
Exaggerates deviations from the Michaelis-Menten kinetics



Kinetic Parameter Determinations

- Michaelis-Menten Kinetics (Simple form)
- Rate of Metabolism, $v = \frac{V_{\max} * S}{K_m + S}$
- $CL_{\text{int}} = V_{\max} / K_m$ (mL/min*mg) for simple Michaelis-Menten Kinetics
- 10 substrate concentrations (cover above and below K_m)
- Determine metabolite formation under linear conditions for time and protein
 - < 20% substrate utilization

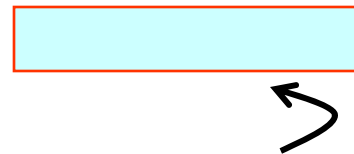


Examples of Common Drugs

Drug	CL [mL min ⁻¹ kg ⁻¹]	Classification
Diazepam	0.38	Low
Theophylline	0.65	Low
Caffeine	1.4	Low
Midazolam	6.6	Medium
Felodipine	12	Medium
Propranolol	16	High
Nitredipine	21	High

Relative Activity Factor and ISEF for Predictions with rCYPs

- Recombinant CYP activity cannot be compared to HLM activity directly...need a conversion factor
- RAF (relative activity factor) and ISEF (inter system extrapolation factor) convert rCYP activity to activity in HLM
 - $CL_{rCYP,int}$ to $CL_{H,int}$
- RAF
 - HLM probe/rCYP probe
 - Specific activity / Turnover Number
 - RAF units are pmol/mg
- ISEF
 - Takes into account CYP abundance in reference HLM pool (e.g. BD Ultrapool™ HLM 150 donor pool)
 - $HLM_{probe}/(rCYP_{probe} \times CYP \text{ abundance})$
 - ISEF have no units
 - Use Cl_{int} for HLM and rCYP (V_{max}/K_m) vs V_{max} activity
 - Drug XYZ $CL_{H,int} = ISEF \times V_{max}/K_m \times CYP \text{ abundance} \times \text{Liver Scaling Factors}$
- ISEF allow population variability to be accessed



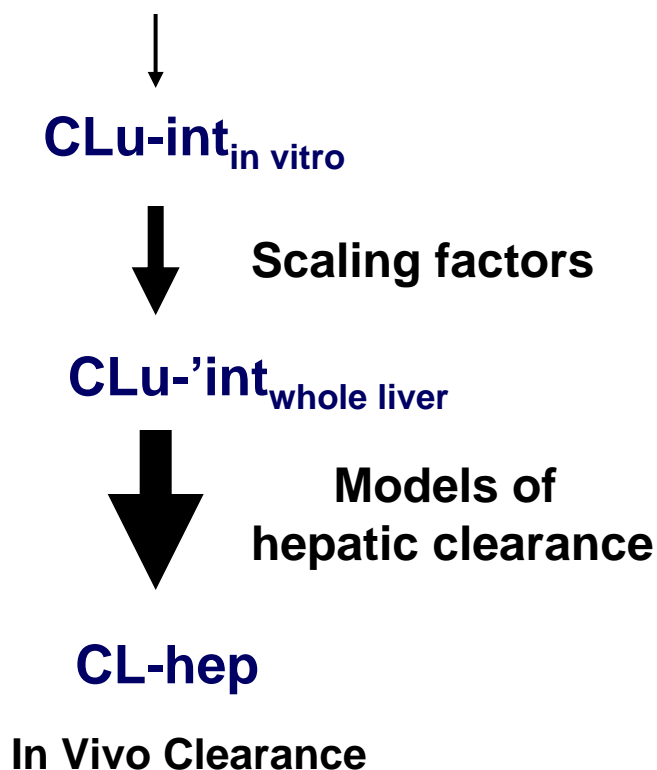
rCYP vs HLM and Hepatocytes for Predicting In Vivo CL

Data from Stringer, et al. DMD, 2009

	rCYP	HLM	Hepatocytes
N	72	41	57
% Inside 2-fold error	32	29	18
% Inside 5-fold error	73	66	46



Scaling Intrinsic Clearance to In Vivo Hepatic Clearance



Well-Stirred Model:

$$CL_h = \frac{f_{ub} * CL_{int}'_{in\ vitro} * Q_H}{f_{ub} * CL_{int}'_{in\ vitro} + Q_H}$$

Parallel Tube Model:

$$CL_h = Q_H - Q_H * \exp(-f_{ub} * CL_{int}'_{in\ vitro}/Q_H)$$

Dispersion Model:

$$CL_h = Q_H * \frac{1-4a}{(1+a)^2 * \exp[(a-1)/2Dn] - (1-a)^2 * \exp[-(a+1)/2Dn]}$$

$$a = (1 + 4Rn * Dn)^{1/2}; Dn = 0.17 \text{ (dispersion no.)};$$

$$Rn = f_{ub} * CL_{int}'_{in\ vitro}/Q_H \text{ (efficiency no.)}$$

(*f_{ub}* = fraction unbound; *Q_H* = hepatic blood-flow)



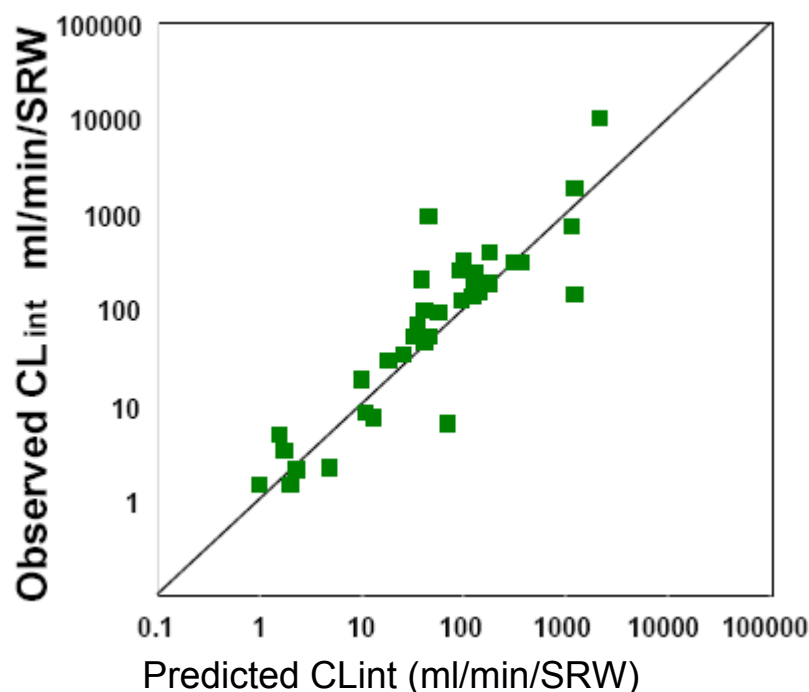
Limitation of Models for Predicting In Vivo Clearance

Clearance predictions in freshly isolated rat hepatocytes and human cryopreserved hepatocytes

Rat hepatocytes

High in vitro – in vivo correlation (fresh)

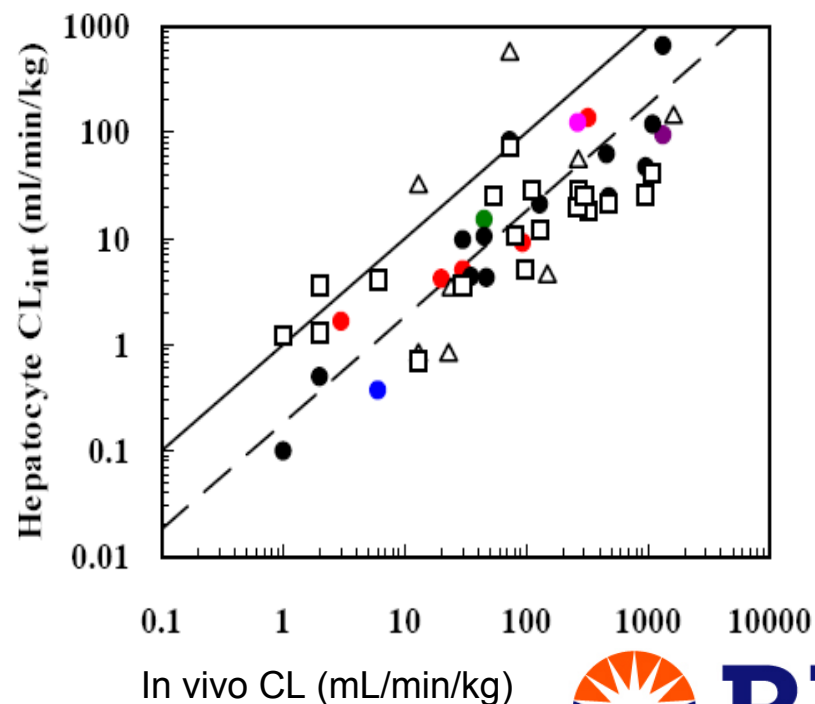
Decreased correlation with frozen cells



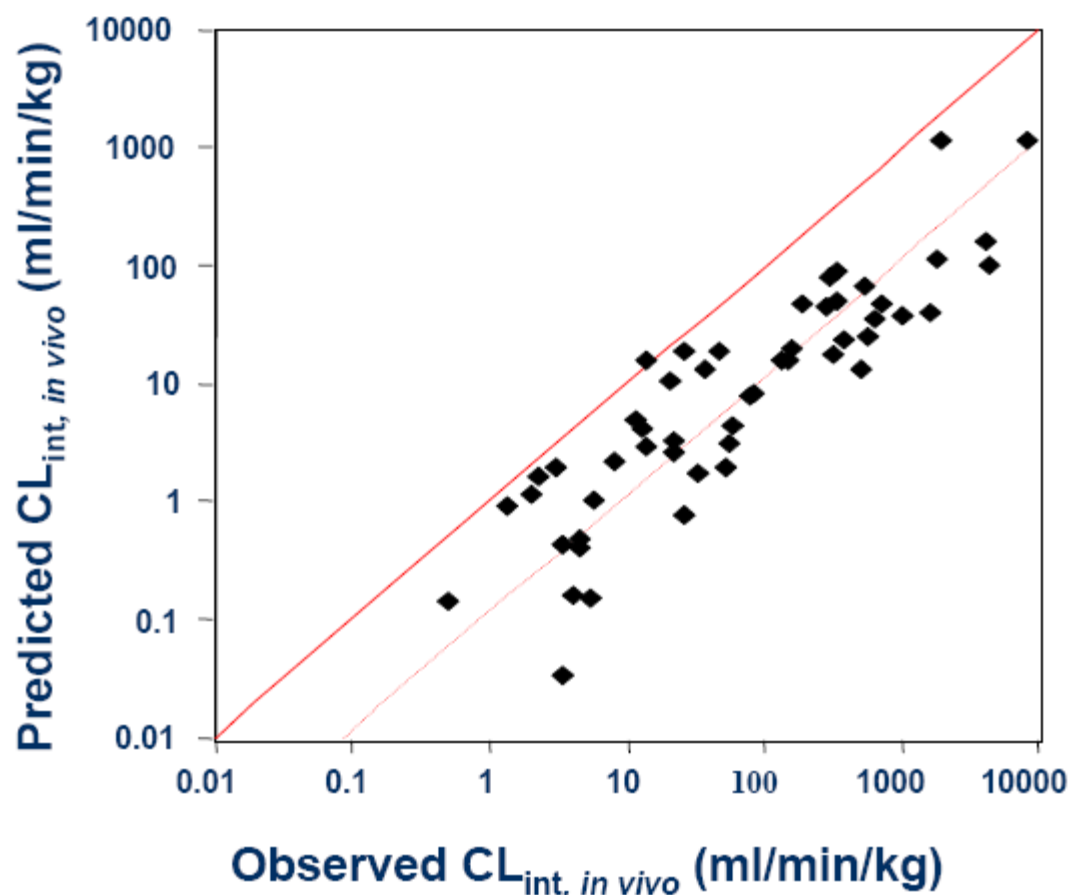
Human cryopreserved hepatocytes

~4.2 fold under prediction

(Brown et al., 2006)



Prediction of Clearance Using Human Liver Microsomes (Ito, et al. DMD, 2005)



- 52 drugs from 5 studies
- Loss of parent and metabolite formation

Systemic Under Prediction

Summary of Prediction Reliability

- Models tend to under predict for human Clearance (Chiba, et al. 2009)
 - Microsomes: ~9-fold under prediction
 - Hepatocytes: ~3 to 6-fold under prediction
- Fresh prepared rat hepatocytes gives most reliable predictions (good correlation between *in vitro* and *in vivo* CL)
- Human hepatocytes give more reliable predictions vs HLM
- Predictions most unreliable for stable, low CL drugs—low CL *in vitro*, but high CL *in vivo* (model systems works best for high CL drugs)
- Possible explanations for discrepancy
 - Damage to metabolic enzymes during tissue handling
 - Extra hepatic metabolism (gut CYP3A4)
 - Non-specific binding to microsomal lipids and cellular components in the incubation
 - Hepatic drug uptake transporters (concentration in liver > plasma concentration). Measured in hepatocytes by “media loss” assay Oil Centrifugation method.
 - Fatty acid or other inhibitors in media (CYP2C9, UGT2B7)
 - Latency issue for UGTs
 - Incubation of human cryo hepatocytes in 100% serum improved predictions (Blanchard et al., JPP, 2006)
 - CL with serum predicted 85% of compounds within 2-fold (vs 77% without)
 - rCYP will have same under predicting issues if activity related to HLM



Conclusion

- In vitro metabolic stability is important early ADME test for predicting in vivo CL
- Amenable to high throughput screening and automation
- Models for predicting in vivo CL are improving, but still tend to under predict
- General trend is that compounds are becoming more stable and involve more non-CYP pathways for metabolism



Contact Us



Questions?

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