Reaction Phenotyping Methods using Recombinant Enzymes and HLM

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Todays Presentation

- Introduction
  - Drug Metabolism overview
  - *In Vitro* Model Systems
- Reaction Phenotyping

Goal of *In Vitro* ADME Testing: Identify Ideal Drug Candidates early in Development Process

Ideal Drug from 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**

Phase I and Phase II Biotransformation

Metabolism/Biotransformation is divided into two groups

Phase I: Addition or unmasking of functional, polar moiety (CYP, FMO, MAO, AO)

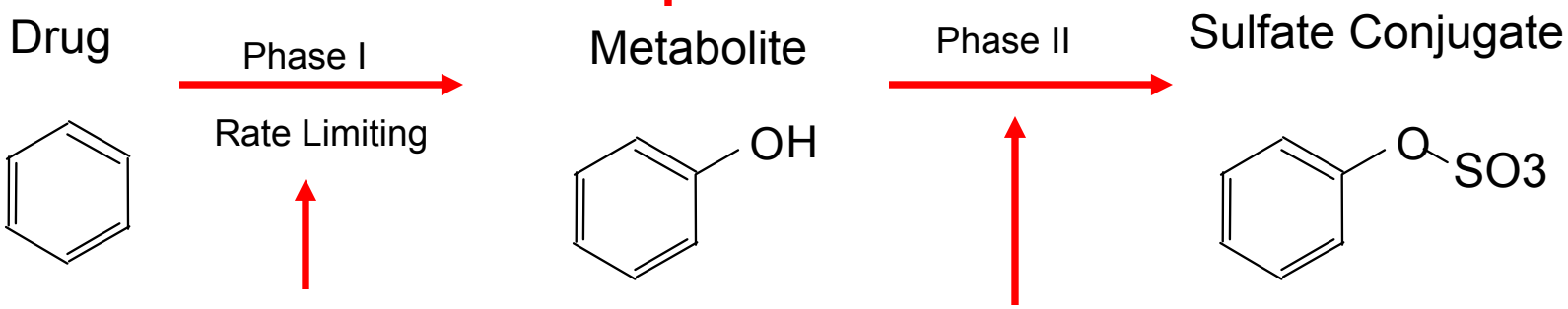
- Oxidation and/or Reduction
- Hydrolysis
- Most typical is Hydroxyl group created or exposed

Phase II: Conjugation with small, endogenous substance, often takes advantage of functional group added in Phase I

End Result: increase polarity and aqueous solubility of drug which facilitates elimination from the body

Phase I and Phase II Metabolism

- Can be active or inactive at target site
- Toxic
- Mutagen or Carcinogen



- Expose functional group that can be conjugate
- Small increase in hydrophilicity

- Large increase in hydrophilicity
- Conjugates are generally inactive

Sites of Drug Metabolism

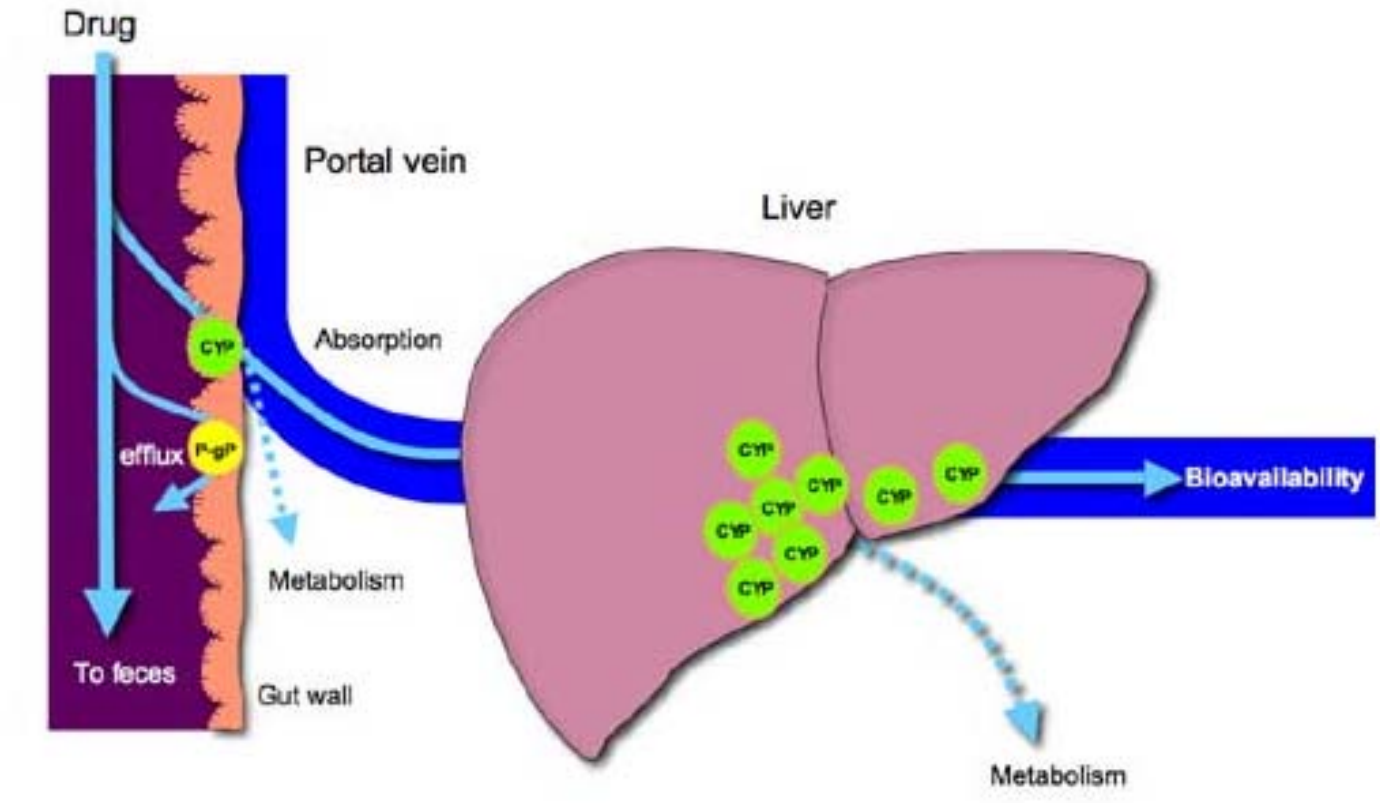
Primary site of Metabolism is Liver

- 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
  - CYPs profile in extra-hepatic tissues is often different than liver

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)

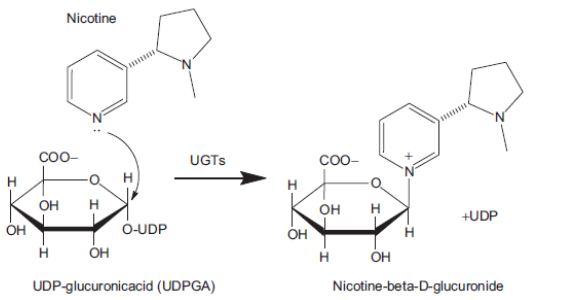


P450s Important for Drug Metabolism

- >60% of drugs on market are metabolized by P450s
- P450 pathway is often rate limiting pathway
- Families 1-3 are the Drug Metabolism CYPs
- “Big 7” CYPs: 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4
- Second tier CYPs
    - 2A6, 2E1, 4F(2/3/12), 2J2, 4A11

UDP-Glucuronosyltransferases (UGTs)

- Most important Phase II drug metabolizing enzyme
- UGTs conjugate glucuronic acid to lipophilic substrates to more water-soluble metabolites, glucuronides, to facilitate excretion
- Glucuronidation rxns:
  - O-glucuronidation
  - N-glucuronidation
  - Acyl-glucuronidation (NSAID)
- UGT Isoforms
  - Hepatic: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7, UGT2B15 and UGT2B10
  - GI specific: UGT1A7, UGT1A8 and UGT1A10



Model Systems for Predicting Metabolic Pathways

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 (-80ºC)
- Withstand several Freeze/Thaw cycles
- Can make Donor Pool; 20 to 150 donors (average patient in population)
- BD UltraPool™ HLM 150
  - 150 donors
    - **Average patient**
    - **Very low lot to lot variability**
  - Equal gender ratio
  - Adult donors only (no pediatrics)
  - Pool contains equal amounts of microsomal protein from each donor
  - Pool quality livers not used (based on P450 spectra; low P420 content)
- 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, AO/XO, ADHs, NATs)
- Same advantages and uses as HLM
- P450 activity ~five-fold lower vs HLM

In Vitro Systems

V. Recombinant Enzymes

- Single DME expressed in a cell line
  - Baculovirus-insect cells (BD Supersomes™)
  - 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

Reaction Phenotyping Methods

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Reaction Phenotyping

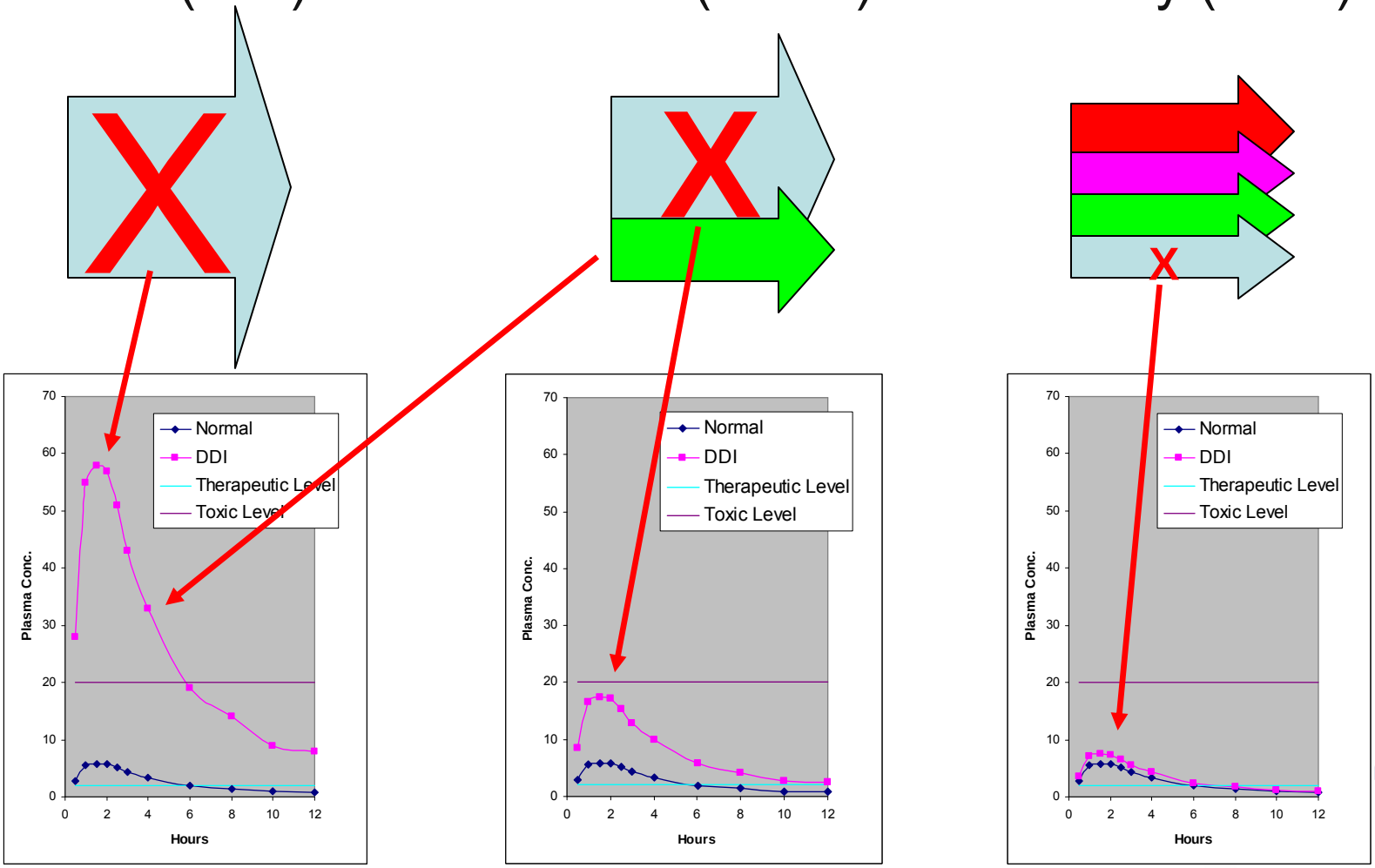
- Pharmaceutical companies are required to characterize all the routes of clearance (drug elimination)
- Why? Because impairment of a route of elimination pathway (by a DDI, polymorphism or disease) can elevate drug levels and cause toxicity
- Generally, pharmaceutical companies are looking for drugs with multiple routes of elimination
  - If any one route is impaired, the others can compensate
- Reaction phenotyping measures the proportion of metabolism elimination which is carried out by the different enzymes
- Requires a sophisticated experimental approach
- Three basic approaches; approach used will depend on development stage

Reduce chance of Drug Candidate becoming a Victim – Increase the Number of Routes of Clearance

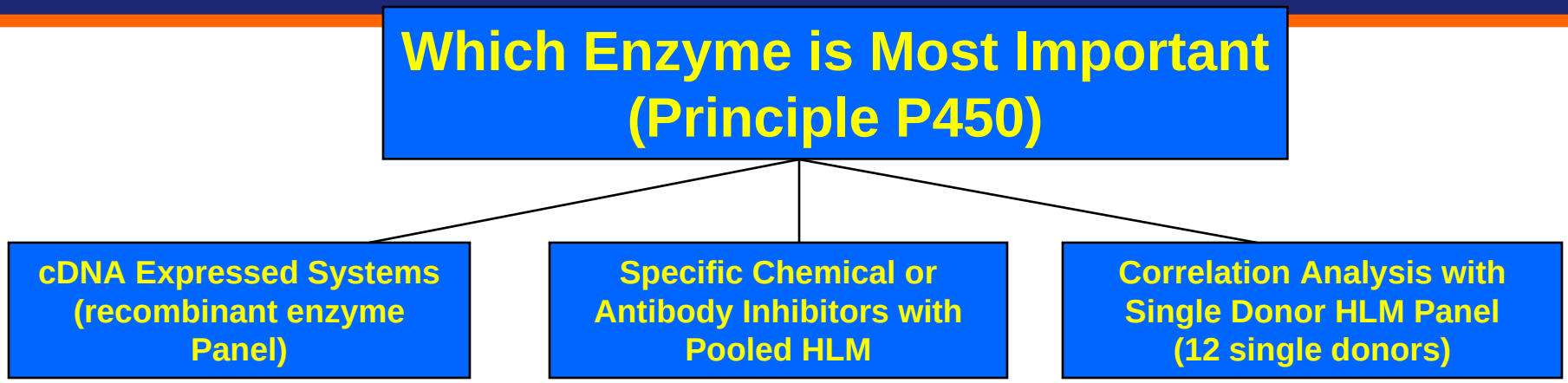
One (bad)

Two (better)

Many (ideal)



Integrated Approach to Reaction Phenotyping



- Incubations with panel of recombinant CYPs to identify important P450s. Use Relative Activity Factors (RAFs) approach and/or relative hepatic abundance of the enzymes to determine relative importance when multiple CYPs involved. Scaled to predict *in vivo* clearance.
- The effect of co-incubated CYP-selective chemical or monoclonal antibody inhibitors on rates of metabolism in HLM can be used to identify primary DMEs
- A correlation of rate of metabolism can be made with a panel of HLM donors ($n \geq 10$) that have been phenotyped for the major DMEs
- Each can have own limitations – combined approach is typically employed

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Reaction Phenotyping in Drug Development Stages

- Reaction phenotyping conducted at multiple stages during drug development process
- Approaches will vary depending on stage (and specific laboratory preferences)
- **Discovery Stage**
  - Loss of parent assay method at a low drug concentration (~1 µM); radiolabelled compounds not available for metabolite quantitation
  - Typically involves high-throughput systems (multi-well formats)
  - Generally use single method Rx Phenotyping approach; limit testing to major CYPs
    - Pooled HLM with selected chemical/antibody inhibitors, or
    - cDNA expressed enzyme panel (in some labs cDNA panel is first tier approach)
- **Early Development Stage**
  - Expand studies with HLM and cDNA-expressed CYPs; use comprehensive panels of inhibitors and/or cDNA-expressed enzymes. Run tests at low drug concentration (single concentration).
  - Measure metabolite formation; identify major metabolites; use radiolabelled test compounds
  - Identify major CYPs for each major metabolite
  - Determine if any Polymorphic CYPs are involved
- **Full Development Stage**
  - Determine full kinetics in HLM (Km and Vmax); metabolite formation with radiolabelled compound
  - Consider non-specific binding in microsomes (fu)
  - Detailed Rx Phenotyping studies, focus on major CYPs (>25% of clearance pathway), include all 3 methods
    - Inhibition in HLM
    - cDNA-expressed enzyme panel
    - Correlation analysis with single donor HLM panel

Common First Step: Determine CYP/non-CYP Involvement

- Flavin Containing Monoxygenase (FMO): microsomal enzyme, dependant on NADPH and O_2 for activity (same as CYP)
  - Both CYP and FMO catalyze hetero-atom oxidation (N- and S-oxidation)
  - C-oxidations carried out by CYP, not FMO
- Aldehyde Oxidase: cytosolic and do not require co-factor
  - Can carry out similar reactions as CYPs
  - Measure activity in HLM vs cytosol and/or S9 (activity in absence of co-factor, and cytosolic > LM)
- Monoamine Oxidase (MAO-A/B): both forms abundant in liver mitochondria
  - No co-factor requirements, both inhibited by Pargyline

Methods to rule out non-CYP oxidative pathways

- 1-Aminobenzotriazole (ABT) is a general CYP inhibitor that can be used to distinguish CYP from non-CYP pathways
  - ~1 mM ABT pre-incubated with HLM, with and without NADPH
  - CYP activity is decreased by 1-ABT in the presence of NADPH
  - Inhibition is not equal among CYPs (most potent for CYP3A4, least for CYP2C9)
  - 1-ABT can be used to inactivate CYPs in hepatocytes
- FMO vs CYP
  - FMO are heat labile in absence of NADPH: pre-heat HLM to 45°C for 1 minute with/without NADPH
  - In absence of NADPH heat will inactivate FMO, but not CYP
  - Non-Ionic detergent inactivation of CYPs: CYPs are sensitive to non-ionic detergents (1% Triton X-100), while FMOs are resistant
  - FMOs not inhibited by general CYP inhibitors: e.g. 1-ABT (methimazole inhibits FMO, but also several CYPs)

Establishing Linear Assay Conditions

- Determine linear assay conditions for time and protein concentration in HLM
  - Establish using pooled HLM
- Test linearity using multiple time points (e.g. 5 to 60 min) and protein concentrations (e.g. 0.2, 0.4 and 0.8 mg/mL) at multiple substrate concentrations (e.g. 1, 10 and 100 µM)
- Hold one parameter constant (e.g. protein) and vary the other (time), and test at each substrate concentration
- Repeat the process holding the other parameter constant
- The linear assay conditions established for pooled HLM will be used for all future studies involving HLM: Kinetic analysis and phenotyping with HLM (inhibition and correlation analysis)

Substrate Concentration Considerations

- In Discovery Stage kinetic parameters are not known; typically start with low substrate concentration and measure loss of parent
- Kinetic parameters (Km and Vmax) for HLM (pooled) are typically determined in development. Measure metabolite(s) formation with radiolabeled test compounds.
- Assay needs to be carried out under linear conditions for both time and protein concentration
  - Substrate utilization <10% if possible (can be difficult at low [S]), not to exceed 20%.
  - May have to re-adjust time or protein to achieve low substrate utilization
  - Adjust for non-specific binding (determine free-fraction); extensive binding (lipophilic compounds) will decrease effective concentration and increase Km
- Typically use 12 to 16 substrate concentrations: 0.1xKm to 10xKm
  - Initial Km can be estimated from linearity studies
- Plot data to obtain kinetic parameters: Km & Vmax
  - Determine if single or multiple Km values
  - Simple Michaelis-Menten or Allosteric Kinetics (S_{50})
  - Intrinsic Clearance (Clint) = Vmax/Km (mL/min/mg HLM)

P450 Enzyme Kinetic Plots

Michaelis-Menten Kinetics

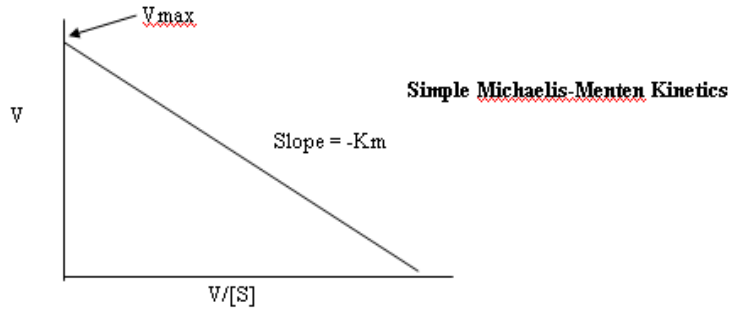
$$v = \frac{Vmax * S}{Km + S}$$

Linear Transformation

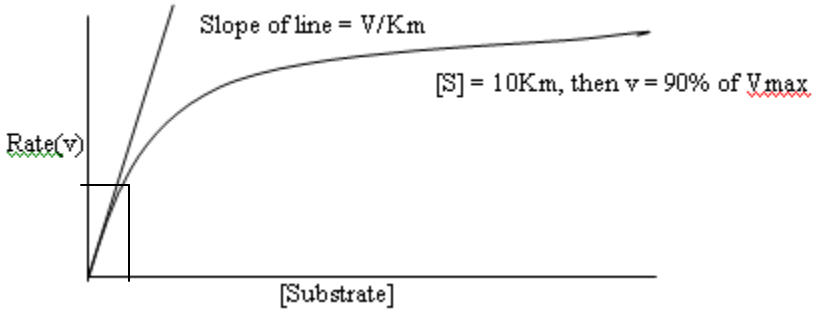
Eadie-Hofstee Plot: v = Vmax - Km (v/[S])
Exaggerates deviations from the Michaelis-Menten kinetics

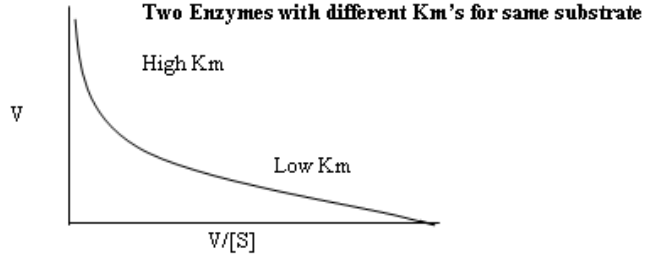
Rectangular Hyperbola

Direct Plot: V vs [S]

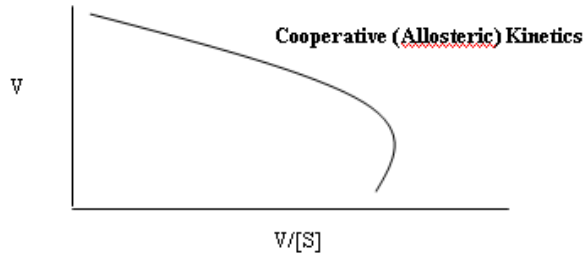


V = Vmax





Rx Phen at two [S]; high and low



Km: substrate concentration that gives ½ Vmax

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Reaction Phenotyping with Chemical and Antibody Inhibitors ("knock-out" method)

- Enzyme source: pooled HLM (represent average patient), e.g. BD UltraPool™ HLM 150
- Substrate concentration: pharmacologically relevant [S]
  - May require 2 concentrations (high and low) if multiple CYPs involved (high/low Km isoforms)
  - If *in vivo* concentration is not known, then [S] < Km
  - Common [S] in absence of kinetic or *in vivo* data is 1 µM (typically below Km, first-order reaction kinetics)
- CYPs can be inhibited by common solvents used to dissolve test compounds and inhibitors
  - Keep solvent concentrations to a minimum: Methanol (<1%), ACN (<2%) and DMSO (<0.2%)
  - Include solvent negative controls for chemical inhibitors
- Negative controls for antibodies: Pre-immune serum or ascites, or irrelevant antibody
  - Antibodies are seldom purified and contain albumin; may result in non-specific binding of test compound or other artifacts

Chemical Inhibitors

- Chemical inhibitors are typically inexpensive and easy to use
- Selective, potent inhibitors not available for all CYP isoforms (e.g. inhibitors to distinguish CYP3A4 & 3A5 involvement)
- Can have a narrow range where potency and specificity are at a maximum
  - E.g. Ketoconazole, CYP3A4 inhibitor, can inhibit other CYPs at low micromolar concentrations: CYP4F2/12/3B, 2C8/9, 2B6, 2J2, 1A1 and 1B1
  - Azamulin shown to be more selective vs KTZ (Stresser et.al.)
- Mechanism of inhibition needs to be considered (competitive, non competitive, time dependent [increased potency over time)
  - Competitive inhibitor potency depends on [S]
  - TDI requires pre-incubation with NADPH
- Chemical inhibitors can be subject to metabolism/depletion in HLM; reduced potency over time
- Inhibitor potency may be substrate-dependent (IC_{50} varies across substrates)
- Inhibitor of one CYP may activate another; e.g. α-naphthoflavone potent inhibitor of CYP1A2, but can activate CYP3A4

CYP Inhibitors (Zhang, Expert Opinion, Review, 2007)

CYP form(s)	Inhibitor	Inhibitor concentrations
CYP1A2	Furafylline α-Naphthoflavone	10 – 30 μM* 1 μM
CYP2A6	Methoxsalen	1 μM
CYP2B6	ThioTEPA	50 μM
CYP2C8	Montelukast	0.1 μM‡
CYP2C9	Sulfaphenazole	10 μM
CYP2C19	Benzylnirvanol	1 μM
CYP2D6 CYP2D6 and CYP3A4/5	Quinidine	< 2 μM 10 μM
CYP2E1	Diethyldithiocarbamate	50 μM
CYP3A4/5 and others	Ketoconazole	1 and 10 μM
CYP3A4/5	Troleandomycin	50 μM*

*Inhibition potency increases with preincubation in the presence of NADPH (time-dependent inhibitors). A 15- to 30-min preincubation is recommended.
‡Montelukast is a potent CYP2C8 inhibitor. However, protein binding is a significant factor and concentrations of the inhibitor should be adjusted for liver microsomal protein content.

Inhibitory Antibodies

- Commonly used in Reaction Phenotyping Studies
- Complete panel to cover all isoforms not available
- Can be more specific than chemical inhibitors (i.e. monoclonals and peptide antibodies)
- Often more costly vs chemical inhibitor
- Mechanism of inhibition is non-competitive; lowers Vmax (Qin Mei et al, JPET, 1999 & DMD,2002)
  - IH-Abs inhibit independent of test compound concentration (chemical inhibitor potency/selectivity can be effected by substrate and substrate-concentration)
- Inhibitory UGT, FMO antibodies are not available (Western blot antibodies only)

Inhibitory Antibodies (continued)

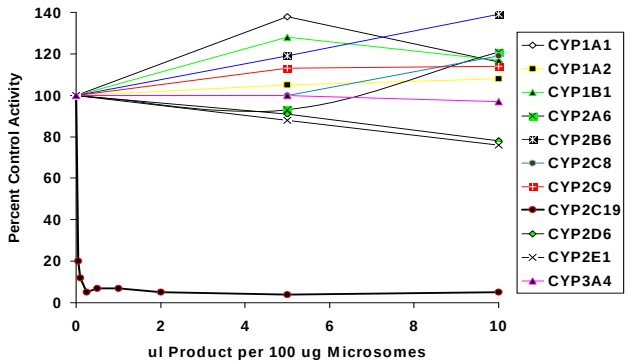
- Majority of commercially available inhibitory CYP antibodies are monoclonals (NIH); single epitope
- Not all IH-MABs are specific (cross-reactivity)
- Incomplete inhibition is somewhat common
  - MAB-CYP/Substrate complex remains productive
  - Can make data interpretation difficult in cases where multiple CYPs are involved
- Peptide antibodies can be made highly specific (pre-determined epitope), however they are typically non-inhibitory…and when they are not all rabbits will produce inhibitory antibodies (need to inject multiple rabbits and screen serum)

Antibody Inhibition Assay Basics

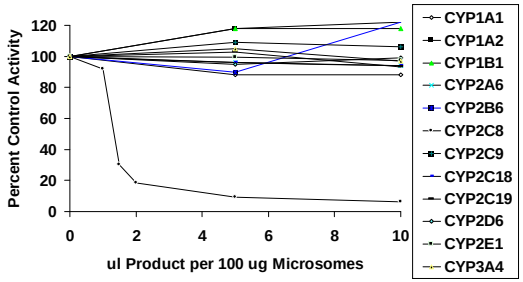
- Antibody should be specific and potent
  - >90% inhibition in recombinant and HLM, otherwise results will be difficult to interpret if multiple CYPs involved
  - Typically the potency is greater in recombinant systems vs HLM
- Should run a titration curve to insure maximal inhibition has been achieved (partial inhibition of a CYP makes interpretation difficult)
- In Theory, if one CYP is involved, ~90% inhibition should be achieved…if only 50% is achieved, then one or more additional CYPs are involved
  - Best case scenario: % Inhibition should add up to ~100%
- A good practice is to conduct initial study in pooled HLM, and follow up with single donors high/low in the CYP of interest (confirmation studies)

CYP2C19 and 2C8 Inhibitory Antibody

Specificity of Inhibition by IH-MAB-2C19



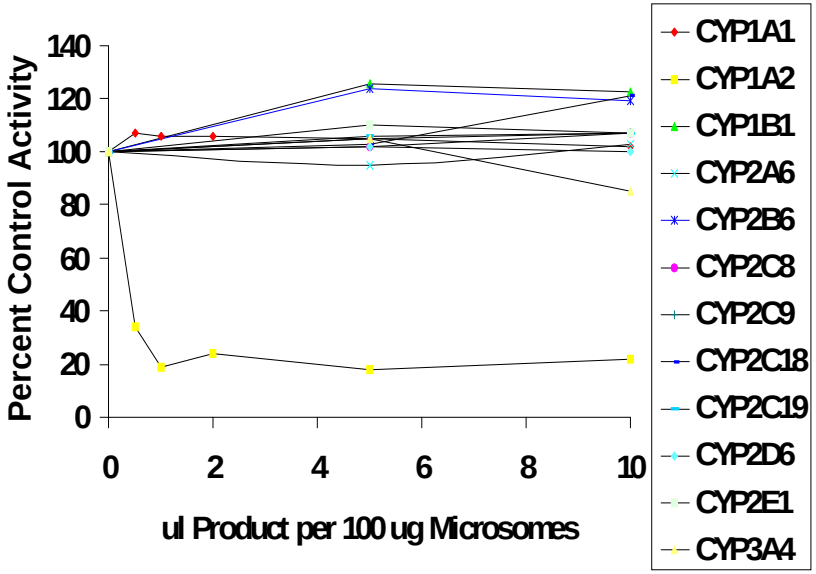
Specificity of Inhibition by IH-MAB-2C8



- Both show high specificity
- >90% inhibition of activity in HLM

CYP1A2 – Inhibitory MAB

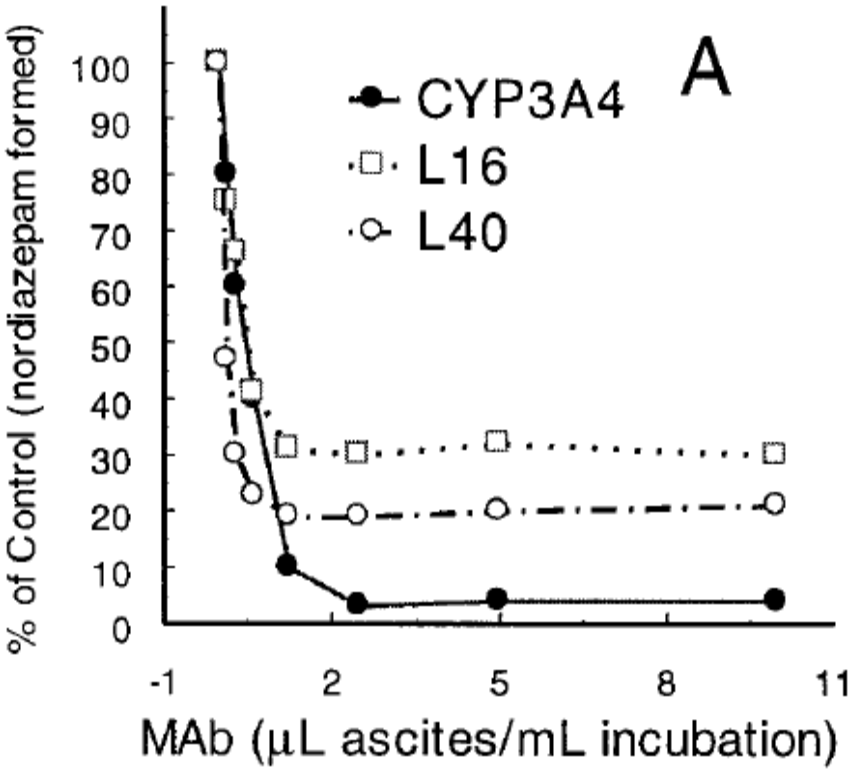
Specificity of Inhibition by MAB-1A2

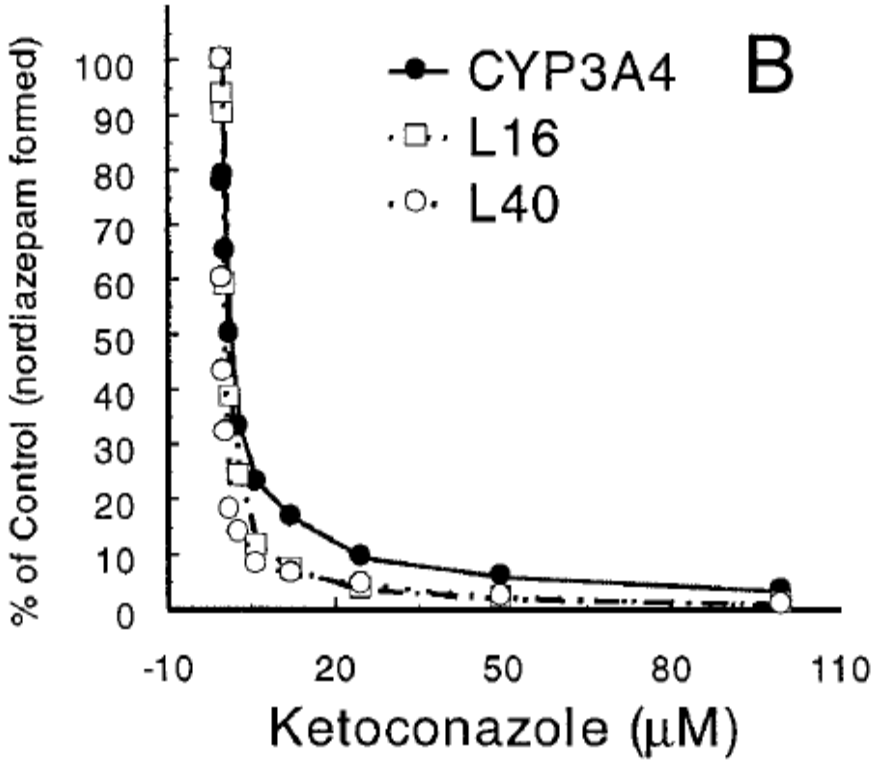


- High specificity for 1A2
- Incomplete inhibition of 1A2 in HLM (~80%)

Antibody vs Chemical Inhibition
(Qin Mei et al., JPET, 1999, vol. 291)

- CYP3A4 contributes only 52 to 73% to Diazepam N-demethylation
- Mab-3A4a (A) detects the involvement of other CYPs
- Ketoconazole selectivity (B) is shown to be concentration dependent (over predicts the contribution of CYP3A4)





BD Gentest™ Chemical/Antibody Reagents for CYP Reaction Phenotyping Applications

CYP	Probe Substrate	Specifc Inhibitor
CYP1A2	Phenacetin	Furafylline [BD]
CYP2B6	Bupropion [BD]	CYP2B6 Mab [BD]
CYP2A6	Coumarin	CYP2A6 Mab [BD]
CYP2C8	Amodiaquine	Montelukast
CYP2C9	Diclofenac	Sulfaphenazole [BD]
CYP2C9	Tolbutamide	Sulfaphenazole [BD]
CYP2C19	(S)-Mephenytoin	Benzylnirvanol [BD]
CYP2D6	Dextromethorphan	Quinidine
CYP2D6	Bufuralol	Quinidine
CYP3A4	Nifedipine	Ketoconazole [BD] or Azamulin [BD]
CYP3A4	Testosterone	Ketoconazole [BD] or Azamulin [BD]
CYP3A4	Midazolam [BD]	Ketoconazole [BD] or Azamulin [BD]

Correlation Approach

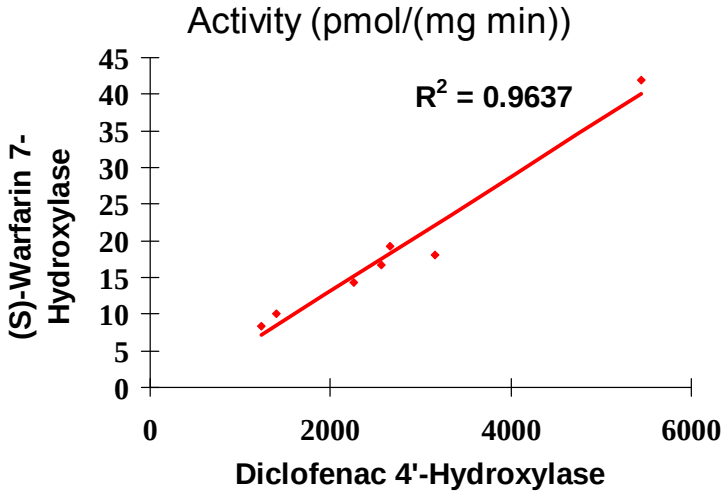
- Takes advantage of wide inter-individual variability of CYP expression levels between donors (>100-fold variability in CYP activity between donors)
- Most direct method – not prone to experimental artifacts from other Rx Phenotyping systems (e.g. chemical/antibody specificity)
- Requires panel of characterized single donor HLM samples; at least 10 single donors
- Panel should have low internal correlation between any two CYPs
- Correlate rates of metabolism of NCE vs CYP-probe substrate activity in panel and/or CYP abundance in panel - unknown & reference activity/abundance
- Labor intensive, typically carried out in development stage. Used to confirm results from HLM inhibition & recombinant CYP studies
- Works best when one enzyme is involved per metabolite
  - Conduct analysis with CYP inhibitors when multiple CYPs suspected
- Need to examine the graph
  - Correlation dependent on single point outlier
  - Check if regression line passes through zero
    - Positive y-axis intercept and high correlation could mean another enzyme is involved

Low Correlation Among Enzyme Activities

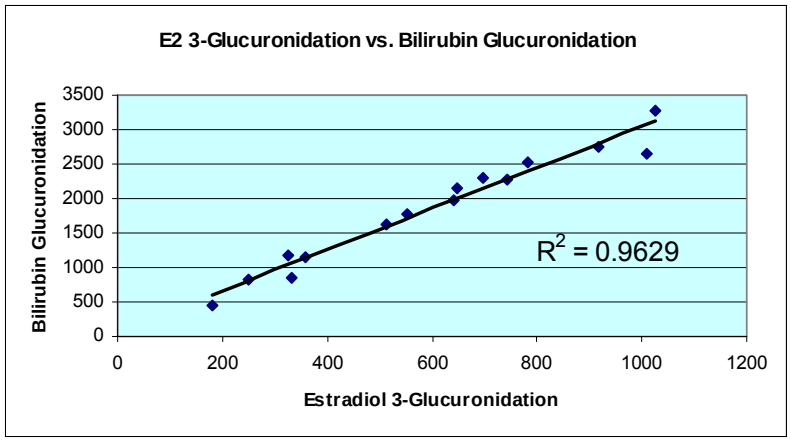
	CYP1A2	CYP2C9	CYP2C19	CYP2B6	CYP2D6	CYP3A4
CYP1A2		0.2446	0.0685	0.0025	-0.3785	0.2627
CYP2C9	0.2446		-0.3924	0.3876	0.1798	0.4786
CYP2C19	0.0685	-0.3924		-0.1748	-0.4706	0.0769
CYP2B6	0.0025	0.3876	-0.1748		0.1844	**0.4683**
CYP2D6	-0.3785	0.1798	-0.4706	0.1844		-0.3600
CYP3A4	0.2627	0.4786	0.0769	0.4683	-0.3600	

UGT-Isoforms	UGT1A1	UGT1A4	UGT1A9
UGT1A1		-0.252	0.317
UGT1A4			0.135
UGT1A9			

Correlation Analysis Examples
CYP2C9 and UGT1A1



CYP2C9 Substrates



UGT1A1 Substrates

Reaction Phenotyping using Recombinant CYPs

- cDNA-Expressed P450s: Single CYP isoform expressed in a cell-based system (BD Supersomes™)
  - Co-expressed with P450 Oxido-Reductase (OR), with or without cytochrome b5
    - b5 can stimulate, inhibit or have no effect depending on CYP and substrate
- Incubate test compound with a panel of cDNA-expressed enzymes
- Major advantage to cDNA approach is that all the major CYPs and UGT cDNA-expressed enzymes are commercially available (BD Supersomes™), unlike chemical or antibody inhibitors
  - Less common CYPs - CYP4F (2, 3A, 3B, 12), 2J2, 1A1, 1B1, 2C18 - are available as BD Supersomes
  - Easily determine all the important CYPs, regardless of number of isoforms involved
- Accessory proteins (OR, b5), and CYPs, are generally "over-expressed" relative to HLM, producing higher activity in recombinant system vs the same CYP in native HLM environment
  - High activity is an advantage when testing slowly metabolized compounds; HLM may not be activity enough to generate metabolites or show loss of parent
- Activity needs to be normalized to correct for the higher activity vs HLM

BD Supersome™ Characterization

- Gene sequence is perfect match with sequence published at U.S. National Library of Medicine
- Sold as insect cell membrane preparations (CYPs are membrane bound, same as in HLM; retain native membrane structure)
- Co-expressed with P450 OR and in some cases with Cytochrome b5
  - Cytochrome b5 can increase, decrease, or have no effect on activity depending on CYP and test compound
  - In most cases b5 increases Vmax (CYP3A4), but can also effect Km (e.g. CYP2E1; b5 decreases Km)
- Substrate specificity consistent with literature reports
- Michaelis-Menten constant (Km) is generally consistent with value observed in pooled HLM
  - Insures the membrane structure of the Supersome is the same as in native tissue (liver or intestine)
  - CYPs, UGTs and MAOs are all membrane bound proteins…NAT is our only soluble Supersome
  - Km: substrate concentration that gives ½ maximal activity (it is a "constant" for a given enzyme)

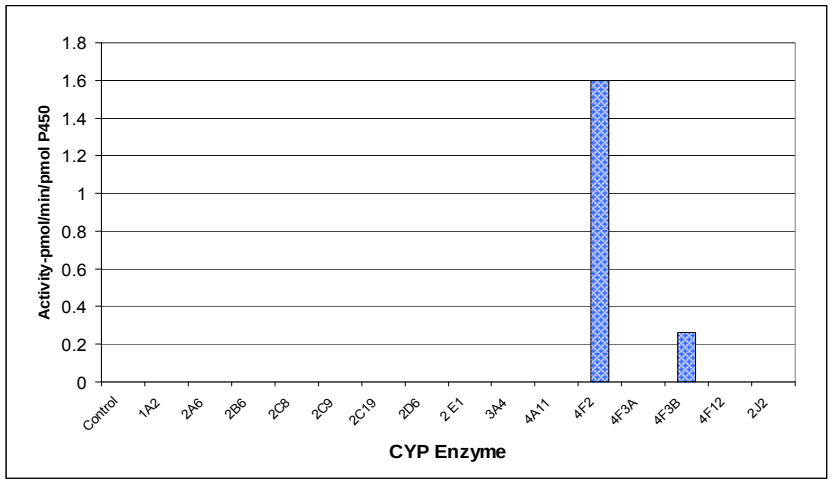
		Km Value (uM)	
Enzyme	**Substrate**	**Supersomes**	**Tissue Fraction**
CYP3A4	Terfenadine	1.9	2.0
CYP2C9	Diclofenac	4.2	2.9
UGT1A1	Estradiol	30	33
UGT1A4	Trifluperazine	100	90
UGT-1A9	Propofol	21	19
MAO-A	Serotonin	86	91

Provides assurance that it is the right protein behaving the right way

Involvement of Non-Traditional CYPs (CYP4F Metabolism of FTY-720, Yi *et.al.* 2011)

Correlation analysis of fingolimod metabolism rates with marker enzyme activities in a panel of HLM from individual donors

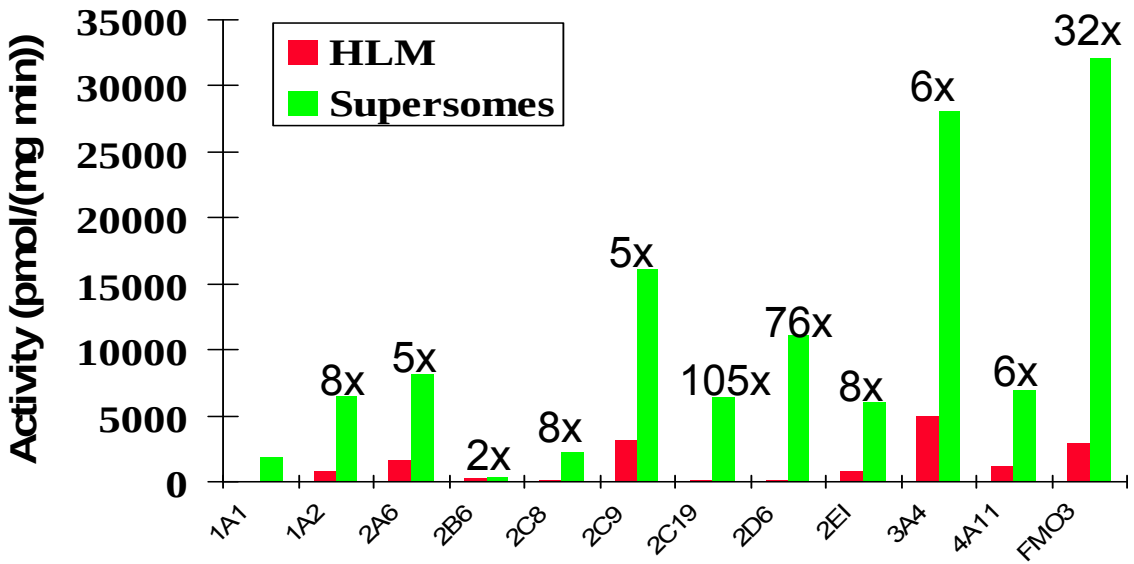
Enzyme	Marker Reaction	Correlation (*R* value)
CYP1A2	Phenacetin *O*-deethylation	0.08
CYP2A6	Coumarin 7-hydroxylation	0.2
CYP2B6	(*S*)-Mephenytoin *N*-demethylation	0.25
CYP2C8	Paclitaxel 6α-hydroxylation	0.18
CYP2C9	Diclofenac 4′-hydroxylation	0.4
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylation	0.03
CYP2D6	Bufuralol 1′-hydroxylation	0.39
CYP2E1	Chlorzoxazone 6-hydroxylation	0.32
CYP3A4/5	Testosterone 6β-hydroxylation	0.32
CYP4A	Lauric acid 12-hydroxylation	0.32
FMO	Methyl *p*-tolyl sulfide oxidation	0.4



- Correlation study was negative for major CYPs
- Available chemical and antibody inhibitors showed no effect
- cDNA panel study: BD Supersomes™, CYP4F2 and 4F3B, were CYPs showing significant activity for the major metabolite (M12)
- CYP4F2 and HLM showed similar kinetics for FTY-720
  - K_m ~ 100 µM, showed substrate inhibition in both systems

BD Supersomes™ versus HLM

- High Expression Level of OR, b5 and CYP
- High Activity (typically several fold higher than HLM)
- Scaling Methods: Normalize rCYP data to accurately predict CYP activity in HLM environment; assign relative contribution when multiple CYPs shown to metabolize test compound
  - Relative abundance approach
    - Multiply BD Superome activity for NCE x CYP abundance in HLM
  - RAF
  - ISEF



When more than one CYP involved, which is most important?

"Relative Activity Factor" Method – RAF (Crespi, C *et.al.*)

- **RAF: Relates the activity of cDNA-expressed enzyme to the activity of the enzyme in its native environment - HLM.**
- **RAF = HLM activity of probe substrate/rCYP activity of probe substrate**
  - **(pmol/min*mg) / (pmol/min*pmol) = pmol CYP/mg**

- Example (RAF): Recombinant enzyme panel shows that two P450 enzymes metabolize NCE (3A4 and 2C9)
  - rCYP3A4 Activity for NCE = 100 pmol/min/pmol
  - rCYP2C9 Activity for NCE = 20 pmol/min/pmol
  - rCYP3A4 Activity for Probe (Testosterone) = 200 pmol/min/pmol
  - rCYP2C9 Activity for Probe (Diclofenac) = 40 pmol/min/pmol
  - 3A4 Testosterone 6ß-hydroxylase in HLM (probe) = 3000 pmol/min/mg
  - 2C9 Diclofenac 4-hydroxylase in HLM (probe) = 1000 pmol/min/mg

- **RAF 3A4 = 3A4 activity for probe in HLM/r3A4 activity for probe = 15 pmol/mg**
  - Rate of 3A4 NCE activity In HLM = RAF3A4 x r3A4 NCE activity = 1500 pmol/min.mg

- **RAF 2C9 = 2C9 activity for probe in HLM/r2C9 activity for probe = 25 pmol/mg**
  - Rate of 2C9 NCE activity In HLM = RAF2C9 x r2C9 NCE activity = 500 pmol/min.mg

RAF (continued)

- **Predicated Total CYP NCE Activity in HLM:**
  - Sum of calculated NCE activity by all P450s involved:

    1500 (3A4) + 500 (2C9) = 2000 pmol/min*mg

- **Percent Contribution of CYP3A4 and CYP2C9 to NCE Activity in HLM:**
  - CYP3A4: 1500/2000 x 100 = 75%
  - CYP2C9: 500/2000 x 100 = 25%

- **Confirm RAF result with P450 specific inhibitory antibodies or chemical inhibitors:**
  - CYP3A4: 1 µM Ketoconazole = ~75% Inhibition in HLM
  - CYP2C9: 2 µM Sulfaphenazole = ~25% Inhibition in HLM

RAF (continued): I-S-E-F

- **ISEF is "Inter System Extrapolation Factor" (simCYP)**

$$\textbf{ISEF} = \frac{\textbf{V}_{max}\ \textbf{or CL}\ \text{(probe-HLM pool)}}{\textbf{V}_{max}\ \textbf{or CL}\ \text{(probe-rCYP) x CYP abundance in HLM pool (WB)}}$$

- ISEF has no units (cancel out): turnover# of CYP in HLM/ turnover # of rCYP
- Takes CYP abundance in HLM into account
- The ISEF is used to estimate NCE Clearance using rCYP (Supersomes) as the enzyme source. Can also estimate DDI severity in different populations
- ISEF allows for population variability to be brought into the model (ethnicity , age or disease state).
- Intrinsic Clearance of NCE for a specific CYP (mL/min*mg):

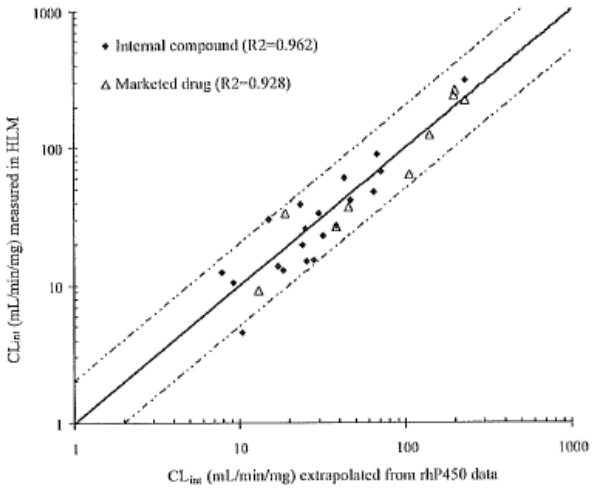
$$\textbf{CL}_{int}\textbf{-NCE} = [\text{CL-}\textbf{ISEF}_{\textbf{CYPx}} \times (V_{max}\text{-NCE [rCYP]}/K_m\text{-NCE [rCYP]}) \times \textit{CYP-abundance}_{(HLM)}]$$

- *CYP-abundance* is where CYP population variability is brought into model
- ISEF should be consistent across all probe substrates for given CYP (work in progress at Simcyp)
- Scale CI_{int} to *in vivo* and hepatic clearance

Qualification of ISEF Method

Chen Y. DMD, 2011

- Studied 20 compounds (10 marketed drugs, 10 in-house compounds)
- Determined ISEF for major CYPs (CYP1A2, 2C8, 2C9, 2C19, 2D6, 3A4)
- Used multiple probe substrates (2C9, 2D6, 3A4)
  - ISEF varies per probe substrate: but were within 2-fold of one another
  - Take average when using multiple probes
- Methods: loss of parent (Cl), metabolite formation, Vmax/Km (Cl)
  - ISEF determined from each method were similar (~within 2-fold)
- Compared ISEF method vs HLM (inhibition) for both Rx Phenotyping and Cl_{int}
  - Tight match between % contribution (ISEF vs HLM)
  - Tight match for Cl_{int} (within 2-fold); Cl-ISEF showed lowest fold error



Conclusion

- FDA requires pathways of elimination identified (≥25% may require in vivo study)
- HLM and recombinant P450s are useful (predictive) models for reaction phenotyping
- An integrated approach involving the 3 methods discussed (or at least 2) is recommended
  - Correlation analysis is being used less; labor intense and interpretation issues when multiple CYPs involved
  - “Knock-out” in HLM and rCYP methods currently the most common
- All major (and most minor) recombinant CYPs available for Rx Phen testing; data should be normalized using either RAF or ISEF
- Antibody and chemical inhibitors are available for major CYPs, but not for less common CYP
  - Room for improvement in chemical/antibody specificity for major CYP inhibitors (more reliable data interpretation)
- Tools for mapping non-CYP metabolic enzymes and transporter pathways are still somewhat limited
- General trend is that compounds are becoming more stable and involve more non-CYP pathways for metabolism

Contact Us

Questions?

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Supplemental Slides

- BD Supersome™ Portfolio
- Scaling Factors
- Scaling Intrinsic Clearance
- Azamulin Specificity vs Ketoconazole

Human BD Supersomes™ Product Portfolio

BD Supersome	Cytochrome b5
CYP1A1	
CYP1A2	
CYP1B1	
CYP2A6	(b_5)
CYP2A13	
CYP2B6	(b_5)
CYP2C8	(b_5)
CYP2C9	(b_5)
CYP2C9*2	
CYP2C9*3	
CYP2C18	
CYP2C19	(b_5)
CYP2D6	
CYP2E1	(b_5)
CYP2J2	(b_5)
CYP3A4	(b_5)
CYP3A5	(b_5)
CYP3A7	(b_5)
CYP4A11	
CYP4F2	(b_5)
CYP4F3A	(b_5)
CYP4F3B	(b_5)
CYP4F12	(b_5)
CYP19	
CYP51	Custom
Total	**23**

Non-CYP BD Superomes
CES1b (Liver)
CES1c (Liver)
CES2 (Intestine)
UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 *(2B10, 2B28 - Dec. 2011)*
FMO1, FMO3, FMO5
MAO-A, MAO-B
NAT1, NAT2

Hepatic Scaling Factors for Whole Liver Intrinsic Clearance

- Microsmal SF (MSF)
  - 40 mg microsomal protein/gr liver (Hakooz, 2006)
  - BD UltraPool™ HLM 150: 43 mg microsome/gr liver
- Hepatocellularity SF
  - 99 million cells/gr liver (Barter, 2007)
- Liver Weight (LW): 1400 grams
- Body Weight (BW): 70 kg
- 20 grams liver/kg BW

- $CLu_{int\text{-}in\ vivo} = (CLu_{int} \times MSF \times LW) / BW$
- Units = µL/min*kg BW (or mL/min*kg BW)

$CLu_{int\text{-}in\ vivo} = CLu_{int}$ *(Vmax/Km)* x 40mg microsome/gr liver x 20 gr liver/kg BW

Scaling Intrinsic Clearance to *In Vivo* Hepatic Clearance

Vmax/Km or Half-Life (hepatocyte/microsomes/rCYPs)

↓

***CL-int*$_{in\ vitro}$**

↓ **Scaling factors**

CL-int'$_{in\ vivo}$

↓ **Models of hepatic clearance**

CL-hep

***In Vivo* Clearance**

Scaling Factors

- 43 mg mic./gram liver
- 20 g liver/kg b.w.

Well-Stirred Model:

$$CLh = \frac{fub * CLint'_{in\ vivo} * QH}{fub * CLint'_{in\ vivo} + QH}$$

Parallel Tube Model:

$$CLh = QH - QH * \exp(-fub * CLint'_{in\ vivo}/QH)$$

Dispersion Model:

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

$a = (1 + 4Rn * Dn)^{1/2}$; $Dn = 0.17$ (dispersion no.);
$Rn = fub * CLint_{in\ vivo}/QII$ (efficiency no.)

(Fub = fraction unbound; QH = hepatic blood-flow)

Azamulin vs Ketoconazole as a selective CYP3A4 inhibitor (Stresser et.al.)

			IC_{50} (μM)	IC_{50} (μM)	IC_{50} ratio
Enzyme	Enzyme source	Substrate	KTZ	AZA	AZA/KTZ
CYP1A1	BD Supersomes™	BzRes	0.017	> 50	>2900
CYP1A2	BD Supersomes	CEC	> 50	> 200	-
CYP1A2	HLM	Phenacetin	55	> 100	> 1.8
CYP1B1	BD Supersomes	BzRes	5.6	> 50	> 8.9
CYP2A6	BD Supersomes	Coumarin	> 50	> 50	-
CYP2B6	BD Supersomes	EFC	6.3	> 50	> 7.9
CYP2B6	HLM	Bupropion	ND	>50	-
CYP2C8	BD Supersomes	DBF	4.4	41	9.3
CYP2C8	HLM	Paclitaxel	8.6	73	8.5
CYP2C9	BD Supersomes	MFC	5.1	57	11.2
CYP2C9	HLM	Diclofenac	9	> 100	> 11
CYP2C19	BD Supersomes	CEC	13	47	3.6
CYP2C19	HLM	S-mephenytoin	57	> 100	> 1.8
CYP2D6	BD Supersomes	AMMC	ND	> 200	-
CYP2D6	HLM	Bufuralol	67	> 100	> 1.5
CYP2E1	BD Supersomes	MFC	ND	> 50	-
CYP2E1	HLM	p-Nitrophenol	> 50	> 50	-
CYP2J2	BD Supersomes	Terfenadine	4.6	6.6	1.4
CYP3A4	BD Supersomes	BFC	0.023	0.026	1.1
CYP3A4	BD Supersomes	Terfenadine	< 0.1[e]	0.12	1.6
CYP3A4/5	HLM	Testosterone	0.06	0.24	4.0
CYP3A4/5	HLM	Midazolam	0.04	0.15	3.7
CYP3A5	BD Supersomes	BFC	0.11	0.38	3.5
CYP3A7	BD Supersomes	BFC	0.16	0.34	2.1
CYP4A11	HLM	Lauric acid	> 100	> 100	-
CYP4F2	BD Supersomes	LTB_4	< 1[f]	46	57
CYP4F3b	BD Supersomes	LTB_4	22	>100	> 4.5
CYP4F12	BD Supersomes	Terfenadine	0.74	29	39

- Azamulin (AZA) is a Mechanism-Based Inhibitor of CYP3A4
- Shows enhanced specificity towards CYP3A4 vs KTZ
- KTZ can inhibit several CYPs at low micromolar concentrations (4F, 2C8/9, 2J2, 2B6, 1A1, 1B1)
- AZA can inhibit 2J2 at micromolar concentration; primarily extra-hepatic CYP (intestine)