Today's 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**
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

Drug → Metabolite → Sulfate Conjugate

- Phase I: Rate Limiting
- Phase II: Sulfate Conjugate

- 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
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)
- **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
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
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

Which Enzyme is Most Important (Principle P450)

- 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 \textit{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.
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 O₂ 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

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} \]

Rectangular Hyperbola

Direct Plot: \( V \) vs \([S]\)

\[ V = V_{\text{max}} \]

\[ \text{Slope of line} = \frac{V}{K_m} \]

\([S] = 10K_m\), then \( v = 90\% \) of \( V_{\text{max}} \)

Km: substrate concentration that gives \( \frac{1}{2} V_{\text{max}} \)

Eadie-Hofstee Plot: \( v = V_{\text{max}} - K_m \left( \frac{v}{[S]} \right) \)

Exaggerates deviations from the Michaelis-Menten kinetics

Linear Transformation

Rx Phen at two \([S]\); high and low
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)

<table>
<thead>
<tr>
<th>CYP form(s)</th>
<th>Inhibitor</th>
<th>Inhibitor concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Furafylline</td>
<td>10 – 30 µM</td>
</tr>
<tr>
<td></td>
<td>α-Naphthoflavone</td>
<td>1 µM</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Methoxsalen</td>
<td>1 µM</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>ThioTEPA</td>
<td>50 µM</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Montelukast</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Sulfaphenazole</td>
<td>10 µM</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>BenzylNirvanol</td>
<td>1 µM</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Quinidine</td>
<td>&lt; 2 µM</td>
</tr>
<tr>
<td>CYP2D6 and CYP3A4/5</td>
<td>Diethyldithiocarbamate</td>
<td>50 µM</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Ketoconazole</td>
<td>1 and 10 µM</td>
</tr>
<tr>
<td>CYP3A4/5 and others</td>
<td>Troleandomycin</td>
<td>50 µM</td>
</tr>
</tbody>
</table>

*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

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

Specificity of Inhibition by IH-MAB-2C8
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

<table>
<thead>
<tr>
<th>CYP</th>
<th>Probe Substrate</th>
<th>Specific Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Furafylline $^{BD}$</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion $^{BD}$</td>
<td>CYP2B6 Mab $^{BD}$</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>CYP2A6 Mab $^{BD}$</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>Montelukast</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>Sulfaphenazole $^{BD}$</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>Sulfaphenazole $^{BD}$</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin</td>
<td>BenzylNirvanol $^{BD}$</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Quinidine</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol</td>
<td>Quinidine</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Nifedipine</td>
<td>Ketoconazole $^{BD}$ or Azamulin $^{BD}$</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>Ketoconazole $^{BD}$ or Azamulin $^{BD}$</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam $^{BD}$</td>
<td>Ketoconazole $^{BD}$ or Azamulin $^{BD}$</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th></th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2B6</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
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<tbody>
<tr>
<td>CYP1A2</td>
<td>0.2446</td>
<td>0.0685</td>
<td>0.0025</td>
<td>-0.3785</td>
<td>0.2627</td>
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<tr>
<td>CYP2C9</td>
<td>0.2446</td>
<td>-0.3924</td>
<td>0.3876</td>
<td>0.1798</td>
<td>0.4786</td>
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<tr>
<td>CYP2C19</td>
<td>0.0685</td>
<td>-0.3924</td>
<td>-0.1748</td>
<td>-0.4706</td>
<td>0.0769</td>
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<tr>
<td>CYP2B6</td>
<td>0.0025</td>
<td>0.3876</td>
<td>-0.1748</td>
<td>0.1844</td>
<td>0.4683</td>
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</tr>
<tr>
<td>CYP2D6</td>
<td>-0.3785</td>
<td>0.1798</td>
<td>-0.4706</td>
<td>0.1844</td>
<td>-0.3600</td>
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<tr>
<td>CYP3A4</td>
<td>0.2627</td>
<td>0.4786</td>
<td>0.0769</td>
<td>0.4683</td>
<td>-0.3600</td>
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## UGT-Isoforms

<table>
<thead>
<tr>
<th>UGT-Isoforms</th>
<th>UGT1A1</th>
<th>UGT1A4</th>
<th>UGT1A9</th>
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<tbody>
<tr>
<td>UGT1A1</td>
<td>-0.252</td>
<td>0.317</td>
<td></td>
</tr>
<tr>
<td>UGT1A4</td>
<td></td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td>UGT1A9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Correlation Analysis Examples

**CYP2C9 and UGT1A1**

**CYP2C9 Substrates**

- Diclofenac 4'-Hydroxylase vs. (S)-Warfarin 7'-Hydroxylase
  - $R^2 = 0.9637$

**UGT1A1 Substrates**

- Estradiol 3'-Glucuronidation vs. Bilirubin Glucuronidation
  - $R^2 = 0.9629$
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)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Supersomes</th>
<th>Tissue Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>Terfenadine</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>4.2</td>
<td>2.9</td>
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<tr>
<td>UGT1A1</td>
<td>Estradiol</td>
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<tr>
<td>UGT1A4</td>
<td>Trifluperazine</td>
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<td>90</td>
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<tr>
<td>UGT-1A9</td>
<td>Propofol</td>
<td>21</td>
<td>19</td>
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<tr>
<td>MAO-A</td>
<td>Serotonin</td>
<td>86</td>
<td>91</td>
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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 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
  - $K_m \sim 100 \mu M$, 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

![Activity Comparison Graph](attachment:image.png)

- Activity (pmol/(mg min))
- HLM
- Supersomes

---

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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
  - \(\text{pmol CYP/mg}\)

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
• **Predicated Total CYP NCE Activity in HLM:**
  - Sum of calculated NCE activity by all P450s involved:
    \[ 1500 \text{ (3A4)} + 500 \text{ (2C9)} = 2000 \text{ pmol/min*mg} \]

• **Percent Contribution of CYP3A4 and CYP2C9 to NCE Activity in HLM:**
  - CYP3A4: \[ \frac{1500}{2000} \times 100 = 75\% \]
  - CYP2C9: \[ \frac{500}{2000} \times 100 = 25\% \]

• **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)

  \[
  ISEF = \frac{V_{\text{max or } CL\ (\text{probe-HLM pool})}}{V_{\text{max or } CL\ (\text{probe-rCYP}) \times \text{CYP abundance in HLM pool (WB)}}
  \]

- 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):

  \[
  CL_{\text{int-NCE}} = [CL-ISEF_{\text{CYP}} \times (V_{\text{max-NCE [rCYP]}}/K_{m-NCE [rCYP]})] \times CYP-abundance_{(HLM)}
  \]

  - **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 \(CL_{\text{int}}\) 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 Clint
  – Tight match between % contribution (ISEF vs HLM)
  – Tight match for Clint (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
Questions?

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Supplemental Slides

- BD Supersome™ Portfolio
- Scaling Factors
- Scaling Intrinsic Clearance
- Azamulin Specificity vs Ketoconazole
### Human BD Supersomes™ Product Portfolio

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### 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

- **Microsomal 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**

- \[ \text{CL}_{\text{int-in vivo}} = \left( \text{CL}_{\text{int}} \times \text{MSF} \times \text{LW} \right) / \text{BW} \]
- **Units:** \( \mu\text{L/min*kg BW} \) (or \( \text{mL/min*kg BW} \))

\[ \text{CL}_{\text{int-in vivo}} = \text{CL}_{\text{int}} \left( \frac{V_{\text{max}}}{K_{\text{m}}} \right) \times 40 \text{mg microsome/gr liver} \times 20 \text{ gr liver/kg BW} \]
Scaling Intrinsic Clearance to *In Vivo* Hepatic Clearance

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

\[ \text{CL-int}^{\text{in vitro}} \]

Scaling factors

\[ \text{CL-int}^{\text{in vivo}} \]

Models of hepatic clearance

\[ \text{CL-hep} \]

*In Vivo Clearance*

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

**Well-Stirred Model:**
\[ \text{CL}_{\text{hep}} = \frac{\text{fub} \times \text{CL}_{\text{int}}^{\text{in vivo}} \times \text{QH}}{\text{fub} \times \text{CL}_{\text{int}}^{\text{in vivo}} + \text{QH}} \]

**Parallel Tubular Model:**
\[ \text{CL}_{\text{hep}} = \text{QH} - \text{QH} \times \exp(-\text{fub} \times \text{CL}_{\text{int}}^{\text{in vivo}}/\text{QH}) \]

**Dispersion Model:**
\[ \text{CL}_{\text{hep}} = \frac{\text{QH}}{1 + \frac{1 - 4a}{\exp[(a-1)/2Dn] - (1-a)^2 \times \exp[-(a+1)/2Dn]}} \]

\[ a = (1 + 4 \text{Rn} \times \text{Dn})^{1/2}; \text{Dn} = 0.17 \text{ (dispersion no.)}; \]
\[ \text{Rn} = \text{fub} \times \text{CL}_{\text{int}}^{\text{in vivo}} \text{QII} \text{ (efficiency var.)} \]

(fub = fraction unbound; QH = hepatic blood-flow)
Azamulin vs Ketoconazole as a selective CYP3A4 inhibitor (Stresser et.al.)

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- 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)