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

Timing of Metabolic Stability Testing

• Early Discovery
  – HTS screens for loss of parent
  – Determine in vitro T1/2
  – Rank order compounds, SAR studies
  – Generally test in liver microsomes from rat and human (pools)

• Development
  – Determine $C_{\text{int}}$ with full Kinetics ($V_{\text{max}}$ 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)
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
Phase I and Phase II Drug Metabolism Enzymes in ER and Cytosol

Location of Metabolic Enzymes

Location of UGTs causes “Latency”. Need to add detergent or alamethicin.
Phase I and Phase II Metabolism

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

Drug → Metabolite → Sulfate Conjugate

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

- Large increase in hydrophilicity
- Conjugates are generally inactive
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

Evans and Relling, Science (1999)
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

Evans and Relling, Science (1999)
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
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)
- 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
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Liver tissue

1) Homogenize
2) Low speed centrifugation
3) High speed centrifugation

Supernatant - **Cytosol**

Pellet -- **Microsomes**

Supernatant - **S9**

Pellet -- waste

**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

- CYP1A1-Phenacetin
- CYP1A2-Phenacetin
- CYP2A6-Coumarin
- CYP2B6-7EFC
- CYP2C8-Paclitaxel
- CYP2C9-Diclofenac
- CYP2C19-Mephenytoin
- CYP2D6-Bufuralol
- CYP2E1-p-Nitrophenol
- CYP3A4-Testosterone
- CYP4A11-Lauric Acid
- FMO-3-MTS
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 $Cl_{in}$), 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 µl (~10 µg 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 x (Volume Distribution / 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:
    \[ CL \text{ (Systemic)} = CL_{\text{Hepatic}} + CL_{\text{Renal}} + CL_{\text{Biliary}} + CL_{\text{Other}} \]
Assumptions Required for Predictive Value of Microsomes

- Metabolism is a major mechanism of clearance
  - $\text{CL-met} >> \text{CL-renal} + \text{CL-biliary} + \text{CL-other}$
- Liver metabolic rate $>>$ all other tissues
- Oxidative metabolism predominates
  - $\text{P450} + \text{FMO (UGT)} >>$ 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
  - \( \text{CL}_{\text{int}} = \frac{V_{\text{max}}}{K_m} \) or \( 0.693/t_{1/2} \) (half-life)
    - \( V_{\text{max}} = \text{pmol product/min} \times \text{mg protein} \)
    - \( K_m \) (Michaelis-Menten Constant) = \( \mu \text{M} \)
  - \( \text{CL}_{\text{int}} \) units = \( \mu \text{L/min} \times \text{mg microsomal protein} \) (or # hepatocytes)
Hepatic Scaling Factors for Whole Liver Intrinsic Clearance

- Microsmal SF (MSF)
  - 40 mg microsomal protein/gram liver (Hakooz, 2006)
- Hepatocellularity SF
  - 99 million cells/gram liver (Barter, 2007)

Liver Weight (LW): 1400 grams
Body Weight (BW): 70 kg

\[ \text{CL}_u\text{H,int} = (\text{CL}_{\text{int}} \times \text{MSF} \times \text{LW}) / \text{BW} \]
Units = $\mu$L/min*kg BW (or mL/min*kg BW)
Whole liver CL: \text{CL}_u\text{H,int} and \text{CL}_{\text{int}}......both forms are used

\[ \text{CL}_u\text{H,int} = \text{CL}_{\text{int}} \left( \frac{V_{\text{max}}}{K_m} \right) \times 40 \text{mg microsome/gram liver} \times 1400 \text{ gram liver/kg BW} \]
Liver Models to Determine In Vivo Metabolic Clearance

- Relate drug delivery (controlled by blood flow and drug binding in blood) to the metabolic reaction
- Allow for concentration differences across the liver

Common Assumptions of Liver Models

- Distribution into liver is perfusion rate limited and no diffusional barriers exist
- Only unbound drug crosses the cell membrane and occupies the enzyme site
- A homogenous distribution of hepatic enzymes
Liver Models cont.

Well-Stirred Model
(drug-in = drug-out)

Parallel-Tube Model
(gradient across liver)

Dispersion Model
(represents the two extremes; $D_N$)
Hepatic Clearance (CL_H)
(total body CL = CL_{Heptic} + CL_{Renal})

- Organ Clearance (steady state) = \( Q*(C_{in} - C_{out})/ C_{in} \)
  - \( Q = \) Blood Flow
  - \( C = \) Drug concentration
- Extraction Ratio (ER) = \( (C_{in} - C_{out})/ C_{in} \)
- CL = \( Q * ER \)

\[ CL_H = Q_H * (fu*CLu_{H,int})/(Q_H + fu*CLu_{H,int}), \]
with \[ ER_H = (fu*CLu_{H,int})/(Q_H + fu*CLu_{H,int}), \]
and \[ CL_H = Q_H * ER_H \]
- \( fu = \) 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). Drug concentration coming out of liver equals the intra-cellular drug concentration.

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 devise)
- Estimate from octanol:water partition coefficient (Stringer et al, DMD, 2009)
- \[ CL_H = Q_H * (fu*(CLu_{H,int}/fu_{inc})) / (Q_H + fu*(CLu_{H,int}/fu_{inc})) \]
Hepatic Extraction Ratio

Fraction Escaping Metabolism (Bioavailability (F) = 1 - $E_H$)

Fraction Metabolized ($E_H$)

$CL_H = QH \times E_H = 90 \text{ L/hour} \times 0.9 = 81 \text{ L/hour}$
Flow Limited and Capacity Limited $CL_H$

- **Flow Limited** (*high CL drugs*)
  - $fu \times CL_{uH, int} \gg Q_H$
  - $CL_H = Q_H \times (fu \times CL_{uH, int}) / (fu \times CL_{uH, int}) = Q_H$
  - E.g. propranolol, lidocaine, morphine

- **Capacity Limited** (*low CL drugs*)
  - $fu \times CL_{uH, int} \ll Q_H$
  - $CL_H = Q_H \times (fu \times CL_{uH, int}) / Q_H = fu \times CL_{uH, int}$
  - E.g. warfarin, phenytoin, quinidine, tolbutamide
  - Tolbutamide CL increases in hepatitis patients
    - Due to increase in $fu$ (no change in $CL_{int}$)
**Drug Half-Life Determination**

<table>
<thead>
<tr>
<th>Time (X)</th>
<th>Percent (Y)</th>
<th>LN%</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>4.61</td>
</tr>
<tr>
<td>5</td>
<td>94</td>
<td>4.54</td>
</tr>
<tr>
<td>20</td>
<td>78</td>
<td>4.36</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>3.91</td>
</tr>
<tr>
<td>180</td>
<td>15</td>
<td>2.71</td>
</tr>
<tr>
<td>300</td>
<td>6</td>
<td>1.79</td>
</tr>
</tbody>
</table>

Slope = -k = -0.0115

\( t(1/2) = 0.693/-k = 60.2 \text{ min} \)

- Loss of parent method used most frequently in Discovery Stage (HTS assays)
- Measure disappearance of low concentration of drug (<5 or 10 µM)
- When \( S << K_m \)…\( T1/2 = \ln2/-k \) (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
- \( T1/2 \) units = min - need to include ml/mg into CL\(_{int}\) calculation to convert to mL/min*mg

\[ \text{CLuH\(_{int}\)} = \frac{0.693}{\text{in vitro } T1/2} \times \text{mL incubation/mg protein} \times 40 \text{mg microsome/gram liver} \times 1400 \text{ gram liver/kg BW} \]
Substrate Concentration Plots

Michaelis-Menten Kinetics

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

Rectangular Hyperbola

Direct Plot: \( V \) vs \([S]\) \( V = V_{\text{max}} \)

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

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

Linear Transformation

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

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

- Michaelis-Menten Kinetics (Simple form)
- Rate of Metabolism, \( v = \frac{V_{\text{max}} \cdot S}{K_m + S} \)
- \( CL_{\text{int}} = \frac{V_{\text{max}}}{K_m} \) (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

<table>
<thead>
<tr>
<th>Drug</th>
<th>CL [mL min(^{-1}) kg(^{-1})]</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>0.38</td>
<td>Low</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.65</td>
<td>Low</td>
</tr>
<tr>
<td>Caffeine</td>
<td>1.4</td>
<td>Low</td>
</tr>
<tr>
<td>Midazolam</td>
<td>6.6</td>
<td>Medium</td>
</tr>
<tr>
<td>Felodipine</td>
<td>12</td>
<td>Medium</td>
</tr>
<tr>
<td>Propranolol</td>
<td>16</td>
<td>High</td>
</tr>
<tr>
<td>Nitredepine</td>
<td>21</td>
<td>High</td>
</tr>
</tbody>
</table>
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
  - \( \text{CL}_{\text{rCYP,int}} \) to \( \text{CL}_{\text{H,int}} \)
- 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)
  - \( \frac{\text{HLM}_{\text{probe}}}{(\text{rCYP}_{\text{probe}} \times \text{CYP abundance})} \)
  - ISEF have no units
  - Use \( \text{Cl}_{\text{int}} \) for HLM and rCYP (\( V_{\text{max}}/K_m \)) vs \( V_{\text{max}} \) activity
  - Drug XYZ \( \text{CL}_{\text{H,int}} = \text{ISEF} \times \frac{V_{\text{max}}}{K_m} \times \text{CYP abundance} \times \text{Liver Scaling Factors} \)
- ISEF allow population variability to be accessed
Data from Stringer, et al. DMD, 2009

### rCYP vs HLM and Hepatocytes for Predicting In Vivo CL

<table>
<thead>
<tr>
<th></th>
<th>rCYP</th>
<th>HLM</th>
<th>Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>72</td>
<td>41</td>
<td>57</td>
</tr>
<tr>
<td>% Inside 2-fold error</td>
<td>32</td>
<td>29</td>
<td>18</td>
</tr>
<tr>
<td>% Inside 5-fold error</td>
<td>73</td>
<td>66</td>
<td>46</td>
</tr>
</tbody>
</table>
Scaling Intrinsic Clearance to In Vivo Hepatic Clearance

\[
\text{CLu-int}_{\text{in vitro}} \downarrow \quad \text{Scaling factors} \quad \downarrow \quad \text{CLu-int}_{\text{whole liver}} \quad \downarrow \quad \text{Models of hepatic clearance} \quad \downarrow \quad \text{CL-hep}
\]

\text{In Vivo Clearance}

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

**Parallel Tube Model:**
\[
\text{CLh} = QH - QH \times \exp(-\text{fub} \times \text{CLu-int}_{\text{in vivo}}/QH)
\]

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

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

\(\text{fub} = \text{fraction unbound} ; \quad QH = \text{hepatic blood-flow}\)
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)

- 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
Questions?

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