In vitro mechanistic studies on Metabolism-Dependent Inactivation of Cytochrome P450 Enzymes

Brooke Rock, 2016 DDI Conference, Seattle, WA

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Outline

• Background on mechanism-based (metabolism-dependent) inactivation of cytochrome P450 enzymes

• Importance of screening for metabolism-dependent inactivation in drug discovery

• **Case Study**: AMG 487

• *Old* but new assays to screen for mechanism(s) of metabolism-dependent inactivation
  • Integrating large molecules tools into small molecule assays
Drug-Drug Interactions: Overview

• Drug-Drug Interaction (DDI) can involve:
  • Inhibition of metabolism: resulting in increased pharmacology and potential toxicity
  • Induction metabolism: resulting in decreased pharmacology and potential loss of efficacy

• Clinical consequences of a DDI depend on the therapeutic area and therapeutic window

• Therapeutic areas prone to multiple drug regimens may be less tolerant of new medicines with a DDI liability

• Goal is to understand the potential risk of a DDI before FIH
Definition*: A mechanism-based enzyme inhibitor is a relatively unreactive compound that is activated by an enzyme into a species which, without prior release from the active site, binds covalently to the enzyme rendering it inactive.

Classic features of P450 Inhibition:

- Inactivation is time- and concentration-dependent
- Irreversibility
  1. Heme pyrrole nitrogen alkylation
  2. Protein alkylation
  3. Metabolic intermediate complex (MI complex)
- Hyperbolic kinetics ($K_I$ and $k_{\text{inact}}$)

Mechanism-Based Inactivation Scheme

\[ E + I \overset{k_{on}}{\rightleftharpoons} E\cdot I \overset{k_2}{\longrightarrow} E\cdot I' \overset{k_4}{\longrightarrow} E\cdot I'' \]

\[ E + I' \]

\[ k_{on}, k_2, k_3, k_4 \]

Irreversible inhibition of an enzyme is not simple

Accurate experiments and equations to calculate kinetic constants

Metabolites can greatly confound MDI profiles

Fraction metabolized (fm)

\[ k_{deg} \] of E

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Institute for Scientific Communication
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Goals in Drug Discovery

• Identify and/or create compounds with properties that have a high probability of achieving the desired clinical objectives:
  • Potency-Efficacy
  • Safety
  • Pharmacokinetics
Overview of CYP Inhibition Assays

Application of P450 inhibition assays across the drug discovery---development continuum is dependent upon multiple factors:

Content
  - Usefulness of Information. - Will this information impact decision making?
  - Confidence in Data. - How robust does the information need to be?

EntryPoint
  - Throughput of Assay. - What is the compound pressure for the assay?

Follow-up
  - When will a more rigorous assay be applied to further characterize the lead molecule?

Move faster without loss of data integrity (early 2000s)

Move ever faster with more robust data earlier (current)

Zero tolerance for false negatives in metabolism-based inhibition of CYPs

Integration of multiple experiments lead to a “checked” quadrant

- False Positive: Not ideal but manageable
- True Positive: Prove through mechanism, not just numbers
- True Negative: Difficult to prove
- False Negative: Unacceptable
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Background AMG 487

• AMG 487 is a potent and selective CXCR3 antagonist that exhibited good oral bioavailability and robust in vivo biological activity in a preclinical model of cellular recruitment (Johnson et al., 2007).

• In a single ascending dose phase 1, AMG 487 displayed favorable pharmacokinetics characterized by near-proportional increases in AUC and Cmax exposure over a dose range of 25 to 1100 mg (Floren et al., 2003).

• Unexpectedly, multiple-dose data indicated that nonlinear pharmacokinetic behavior was both time- and dose-dependent, with a key finding that decreasing metabolite/AMG 487 plasma concentration ratios as a function of dose correlated with reduced oral clearance.

• AMG 487 development was subsequently halted as a consequence of these findings and the realization that repeat administration may affect intrinsic clearance of the drug.

Metabolic Scheme of AMG 487

- Minimal MDI potential
- High in vitro clearance
- No GSH adducts detected

Filed in IND

- Potent CYP3A4 inactivator
- Multiple GSH adducts

Determined after FIH study

Metabolites Derived from M2 & M3
Evidence for Cys239 Adduct to CYP3A is the culprit for in vivo inactivation

MS2 spectra
Precursor= [M+H]^3= 522.9
Summary of Findings for AMG 487

- Initial screening of AMG 487 in inhibition assays (TDI or IC50 shift) did not indicate potential for a drug-drug interaction.
- AMG 487 was known to be a high turnover compound in microsomal assay, be on look out for inhibitory metabolites.
- Incorporating multiple screening assays, including longer incubation time with lower enzyme concentrations, AMG 487 exhibited MDI.
- Further evidence of a primary role for a metabolite (M4, tertiary metabolite) in CYP3A4 inhibition was generated by protein labeling and proteolysis experiments, suggesting that a more complex metabolic pathway was responsible for generation of inhibitory metabolites affecting AMG 487 human pharmacokinetics.
- Million dollar question: what additional assays can be applied in drug discovery to improve confidence in a negative and/or positive MDI hits.
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Assays applied to understand CYP inhibition: Integrating large molecules tools into small molecule assays

1. Beyond GSH trapping, utilizing Cys239 on P450 3A4 to trap reactive intermediates
2. Determining $k_{\text{deg}}$ of P450 3A in the presence of different metabolism-dependent inactivators utilizing siRNA technologies
3. Screening for MI Complex via high throughput fluorescent probe screening
Assays applied to understand CYP inhibition: GSH trapping experiments

- Glutathione trapping is often utilized to determine mechanism, assumption is covalent adduct
- Issue: Requires release of the reactive metabolite-intermediate from active site for conjugation


Even with sensitive analytical methods GSH adducts are difficult to detect

Can we exploit nucleophilic residue (Cys 239) within CYP 3A4 protein as a probe for bioactivation?
Assays applied to understand CYP inhibition: Utilizing Cys239 in CYP3A for reactive metabolite trapping

- Incubation in CYP3A4 supersomes™ with test compound at 0.5-50 uM (low protein concentration, 0.25 uM)
- Spin to pellet protein, add digestion buffer, capture with magnetic beads conjugating a specific antibody towards Cys239 peptide
- LC/MS/MS analysis

3A4 Supersome™ mixture

Peptide specific antibody

Performed on Agilent AssayMap Platform

LC/MS
Sciex Triple TOF 5600 Qual/Quant analysis
Assays applied to understand CYP inhibition: Utilizing Cys239 in CYP3A for reactive metabolite trapping

<table>
<thead>
<tr>
<th>Reagent</th>
<th>ID: Cys 239</th>
<th>GSH adduct</th>
</tr>
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<tbody>
<tr>
<td>Maleimide</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Pyrene iodoacetamide</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>ABT (- control)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ritonavir (- control)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Raloxifene (+ control)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>AMG compound 1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>AMG compound 2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>AMG compound 3</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

![Graph showing relative % of unconjugated Cys239 peptide over time](image)
## Important Role of $K_{\text{deg}}$ in MDI predictions

<table>
<thead>
<tr>
<th>Reference</th>
<th>Recommended $k_{\text{deg}}$</th>
<th># Interactions Compared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obach</td>
<td>$0.000321 \text{ min}^{-1}$ $T_{1/2} = 36 \text{ h (1.5 days)}$</td>
<td>10</td>
</tr>
<tr>
<td>Einolf and Xenobiotica</td>
<td>$0.00016 \text{ min}^{-1}$ $T_{1/2} = 72 \text{ h (3 days)}$</td>
<td>100</td>
</tr>
<tr>
<td>Fahmi</td>
<td>$0.0005 \text{ min}^{-1}$ $T_{1/2} = 23.1 \text{ (0.9 days)}$</td>
<td>30</td>
</tr>
<tr>
<td>Galetin and Houston</td>
<td>$0.000128 \text{ min}^{-1}$ $T_{1/2} = 94 \text{ (3.8 days)}$</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### Simplified equation (Mayhew et al, 2000)

$$AUC_i = \frac{1}{AUC} \left( \frac{k_{\text{deg}}}{k_{\text{deg}} + \frac{[I] \times k_{\text{inact}}}{[I] + K_i}} \right)$$

The degradation rate of P450 enzymes is an important variable for predictions.
Multiplexing metabolism dependent screens for P450 enzymes

RapidFire 365: 8 sec/sample
P450 probe activity

Eliminates one mechanism of MDI

Fluorescent plate reader 2 min/plate
P450 probe activity after \([Fe(CN)_6]^{3-}\)

Generates a lot of data quickly; smart interpretation allows for real time SAR to influence design of new chemical entities
Take Home Messages

• Judicial evaluation of compounds in regards to drug-drug interaction potential is not a simple process
  • Tools/technology are always evolving

• Numbers are just numbers. It is important to understand how to measure/determine inhibitory kinetic constants
  • Confirmation of inhibitory potential by determining the mechanism aids in understanding chemical nature/potential of inhibitor

• Drug-drug interactions caused by P450 enzymes are multifaceted, especially in regards to inhibition caused by metabolite-dependent mechanisms
QUESTIONS/DISCUSSION