Strategies for *In Vitro* CYP Inhibition Testing and Alignment with FDA Guidance

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Cytochrome P450 Inhibition

- Major cause of drug drug interactions
- Want to avoid because:
  - Ethical considerations
  - Competition (i.e. for “me too” drug)
  - Product non-approval or withdrawal

POSICOR: 143 Sudden Deaths Did Not Stop Approval

* With study results kept secret, nation got another blood-pressure drug.

By DAVID WILLMAN, Times Staff Writer

Senior FDA officials with the power to approve new drugs were warned in advance about the dangers of Posicor, a pill for high blood pressure and symptomatic chest pain.

The clinical studies of Posicor 'cast a shadow of potential risk for serious arrhythmias,' FDA medical team leader Dr. Shaw T. Chen wrote on Dec. 18, 1996. The data in hand also showed Posicor would interact with certain other drugs, posing potentially severe risk.

A 70-year-old man suffered 'sudden death' in one study of Posicor's effect on chest pain. The senior FDA officials also were told of sudden deaths in 142 other patients who took either Posicor or a placebo in an ongoing study focused on congestive heart failure. However, details from the 2,400-patient study remained sealed because the manufacturer opposed breaking the experiment's confidentiality until it was finished.

This left the FDA officials a choice: Wait a year or more, or approve Posicor without knowing the details.

'I sure don't feel good about what I've seen,' said Dr. Lemuel A. Moya, a member of the FDA's Cardiovascular and Renal Drugs Advisory Committee that met on Feb. 28, 1997. Moya, a physician and biostatistician at the University of Texas, suggested it would be prudent to delay judgment until the study's results were unsealed. 'I'm afraid that we are rushing into this.'

According to a transcript of the meeting, Moya voiced concern about Posicor's effect on heart rhythm and its potential to interact with other compounds. 'Patients will be taking this in fairly uncontrolled situations in combinations of drugs which have ramifications yet unknown,' he said.

Another committee member, Dr. Robert Califf, professor of medicine at Duke University, said: 'If this [drug] was really something that was dramatically different, better than anything else in the way of relieving symptoms, then I would look at it differently. But given the fact there are a lot of other effective therapies out there, why not be safe with the public?'

Indeed, scores of other drugs for treating high blood pressure were already on the market, and Posicor was not proved to offer lifesaving benefit.
Drugs Removed from or Restricted in the U.S. Market Because of Drug Interactions

- Terfenadine (Seldane®) February 1998
- Mibefradil (Posicor®) June 1998
- Astemizole (Hismanal®) July 1999
- Cisapride (Propulsid®) January 2000
- Cerivastatin (Baycol®) August, 2001


- Despite these withdrawals, > 100,000 Adverse Drug Reaction related deaths annually
  - Withdrawals address only the largest and most easily detected ADRs
Metabolism-based Drug-drug interaction - Mechanism

**fixed rate of metabolism**
(e.g. CYP3A4)

**Result**
Therapeutic levels

**“waiting” Victim**
Drug A
Liver
Metabolite of Drug A

Toxic accumulation of Drug A or B

**Victim with perpetrator**
Drug A
Liver
Metabolite of Drug A

Drug B
Liver
Metabolite of Drug B
Presentation overview

• Guidance documents and position papers
• Focus on assessment of CYP inhibition
  – What substrates?
  – Which enzymes?
  – Enzyme source, protein concentration, kinetics, incubation time, analytical considerations
  – Reversible and mechanism-based inhibition
• Strategies for robust analysis
Guidances and position papers

- FDA (April, 1997)
  - Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro
  - sponsored by the FDA, EUFEPS, and AAPS
- FDA (Sept, 2006) DRAFT Guidance for Industry
  - Drug Interaction Studies – Study Design, Data Analysis and Implications for Dosing and Labeling
- Einolf et al (2009) Drug Metab. Dispos – Submitted for publication
  - Time-dependent inhibition consensus paper by PhRMA
FDA Guidance Documents

- > 400 draft or final guidance documents
- Represent the Agency's current thinking
- Do not operate to bind FDA or the public
- An alternative approach may be used if it satisfies requirements of the applicable statute, regulations, or both
- If in doubt, contact the originating office (e.g. CDER)
- Provides Pharma company with assurance
FDA’s current thinking on in vitro studies

“…in vitro studies can frequently serve as a screening mechanism to rule out the importance of a metabolic pathway and the drug-drug interactions that occur through this pathway so that subsequent in vivo testing is unnecessary.”
“if in vitro studies indicate that an investigational drug does not inhibit CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A metabolism, then corresponding in vivo inhibition-based interaction studies of the investigational drug and concomitant medications eliminated by these pathways are not needed.”
Cytochrome P450 inhibition testing in vitro – Basic Concepts

• Why test in vitro?
  – In vitro results predict in vivo result (Obach et al, 2006)
  – Design clinical trial
  – Avoid clinical trial
  – SAR

• Test P450 metabolism of model drug with and without drug candidate (“perpetrator”)
  – Model drug serves a surrogate for all “victim” drugs cleared principally (>0.6 f_m) by that enzyme

• Quantify model drug metabolite formation

• What concentration of drug candidate inhibits reaction? Percent inhibition and/or IC50

Use information to guide decision making
Design Considerations – initial rate conditions

- "We suggest a linear relationship between time and amount of product formed.
- We recommend a linear relationship between amount of enzyme and product formation."

CYP2C19-catalyzed 4'-hydroxylation of S-mephenytoin in HLM
Choice of Substrate

•  “Should be selective
  – predominantly metabolized by a single enzyme in pooled human liver microsomes or recombinant P450s

•  Should have a simple metabolic scheme
  – E.g. no sequential metabolism

•  There are also some practical criteria
  – commercial availability substrate and metabolite(s)
  – assays that are sensitive, rapid, and simple; and a reasonable incubation time”
Which enzymes to test?

- The major CYP enzymes:
  - CYP1A2, 2C8, 2C9, 2C19, 2D6, 3A
- "Drug interactions based on CYP2B6 are emerging as important interactions."
- "Other CYP enzymes, including CYP2A6 and CYP2E1… should be considered when appropriate."
- Microsomal (as opposed to cellular) sources are implied. Human liver or cDNA-expressed.
**Preferred and acceptable chemical substrates for in vitro experiments**

*(9/25/2006)*

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Substrate</th>
<th>Km (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>phenacetin-O-deethylation</td>
<td>1.7-152</td>
<td>7-ethoxyresorufin-O-deethylation</td>
<td>0.18-0.21</td>
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<td>theophylline-N-demethylation</td>
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<td></td>
<td>樱桃碱 1-羟基化</td>
<td></td>
<td>尚可的 1-羟基化</td>
<td>220-1565</td>
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<td>2A6</td>
<td>coumarin-7-hydroxylation nicotine O-oxidation</td>
<td>0.30-2.3</td>
<td>nicotine 1-oxidation</td>
<td>13-162</td>
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<td>2B6</td>
<td>efavirenz hydroxylase</td>
<td>17-23</td>
<td>propofol hydroxylation</td>
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<td></td>
<td>bupropion-hydroxylation</td>
<td>67-168</td>
<td>S-mephenytoin-N-demethylation</td>
<td>1910</td>
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<td>2C8</td>
<td>Taxol 6a-hydroxylation</td>
<td>5.4-19</td>
<td>amodiaquine N-deethylation</td>
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<td></td>
<td></td>
<td>rosiglitazone para-hydroxylation</td>
<td>4.3-7.7</td>
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<td>2C9</td>
<td>tolbutamide methyl-hydroxylation</td>
<td>67-838</td>
<td>flurbiprofen 4'-hydroxylation</td>
<td>6-42</td>
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<td>S-warfarin 7-hydroxylation</td>
<td>1.5-4.5</td>
<td>phenytoin-4-hydroxylation</td>
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<td>diclofenac 4'-hydroxylation</td>
<td>3.4-52</td>
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<tr>
<td>2C19</td>
<td>S-mephenytoin 4'-hydroxylation</td>
<td>13-35</td>
<td>omeprazole 5-hydroxylation</td>
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<td>fluoxetine O-dealkylation</td>
<td>3.7-104</td>
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<td>2D6</td>
<td>(±)-bufuralol 1'-hydroxylation</td>
<td>9-15</td>
<td>debrisoquine 4-hydroxylation</td>
<td>5.6</td>
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<tr>
<td></td>
<td>dextromethorphan O-demethylation</td>
<td>0.44-8.5</td>
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<td>2E1</td>
<td>chlorzoxazone 6-hydroxylation</td>
<td>39-157</td>
<td>p-nitrophenol 3-hydroxylation</td>
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<td>lauric acid 11-hydroxylation</td>
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<td></td>
<td></td>
<td></td>
<td>aniline 4-hydroxylation</td>
<td>6.3-24</td>
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<tr>
<td>3A4/5</td>
<td>midazolam 1-hydroxylation</td>
<td>1-14</td>
<td>erythromycin N-demethylation</td>
<td>33 – 88</td>
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<td>testosterone 6β-hydroxylation</td>
<td>52-94</td>
<td>dextromethorphan N-demethylation</td>
<td>133-710</td>
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<td></td>
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<td>triazolam 4-hydroxylation</td>
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<td>terfenadine C-hydroxylation</td>
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<td></td>
<td></td>
<td></td>
<td>nifedipine oxidation</td>
<td>5.1-47</td>
</tr>
</tbody>
</table>

*Recommend use of 2 structurally unrelated CYP3A4/5 substrates for evaluation of in vitro CYP3A inhibition. If the drug inhibits at least one CYP3A substrate in vitro, then in vivo evaluation is warranted.*
Design Considerations for In Vitro CYP Inhibition Studies – Substrate Concentration for IC50 testing

• “Typical experiments for determining IC50 values involve incubating the substrate, if the metabolic rate is sufficient, at concentrations below its $K_M$ to more closely relate the inhibitor IC50 to its $K_i$”

  – When $S = K_M$…
    • Competitive inhibitors $\rightarrow$ IC50 = 2X Ki
    • Noncompetitive inhibitors $\rightarrow$ IC50 = Ki
  – Regardless, when $S << K_M$…
    • IC50 = $K_i$
IC$_{50}$ values approximate $K_i$ when $S \sim K_M$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>mean IC$_{50}$ (nM)</th>
<th>mean $K_i$ (nM)</th>
<th>Best fit model</th>
<th>ratio IC$_{50}$/K$_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>7,8-Benzoflavone</td>
<td>9</td>
<td>3</td>
<td>Mixed</td>
<td>2.7</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Ketoconazole</td>
<td>2250</td>
<td>1400</td>
<td>Competitive</td>
<td>1.6</td>
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<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>Montelukast</td>
<td>22</td>
<td>13</td>
<td>Competitive</td>
<td>1.7</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>Sulfaphenazole</td>
<td>520</td>
<td>195</td>
<td>Competitive</td>
<td>2.7</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin</td>
<td>(S)-Benzylnirvanol</td>
<td>410</td>
<td>340</td>
<td>Competitive</td>
<td>1.2</td>
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<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Quinidine</td>
<td>62</td>
<td>50</td>
<td>Competitive</td>
<td>1.2</td>
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<td>CYP3A4</td>
<td>Midazolam</td>
<td>Ketoconazole</td>
<td>16</td>
<td>9</td>
<td>Mixed</td>
<td>1.8</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>Ketoconazole</td>
<td>19</td>
<td>21</td>
<td>Competitive</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Values represent means of two independent determinations; Global CV = 0.13

**Mean = 1.7**

**Michaelis-Menten Kinetics**

Assumption of competitive inhibition: If $[S] = K_M$, then IC$_{50} = 2K_i$

Regardless of the mechanism of inhibition: If $[S] \ll K_M$, then IC$_{50} = K_i$
Design considerations – Ki determinations

- “For $K_i$ determinations, both the substrate and inhibitor concentrations should be varied to cover ranges above and below the drug’s $K_M$ and inhibitor’s $K_i$.”
- An appropriate spacing of S and I enhances confidence of the model fit.
- Use $R^2$ and AICc to gauge best fit of model.

Eadie-Hofstee fit:
Quinidine inhibited CYP2D6 catalyzed dextromethorphan O-deethylase
Design Considerations – protein concentration

- “Microsomal protein concentrations used are usually less than 1 mg/ml”
- Excessive concentrations lead to non-specific binding that tends to reduce inhibition response and elevate IC50 values

Effect of microsomal protein concentration and 1 µM ketoconazole on the CYP3A4 catalyzed 10-hydroxylation of warfarin

![Graph showing the effect of microsomal protein concentration on 10-OH Warfarin production](image-url)
Design Considerations – buffers

- “Because buffer strength, type, and pH can all significantly affect Vmax and Km, standardized assay conditions are recommended.”

- Common Variables
  - MgCl₂
  - KP04, NaPO4, Tris-Cl, NaCl, KCl
  - 25 to 200 mM ionic strength

- Specific recommendation are not stated
Effect of salt concentration – the contrast of CYP3A4 and CYP2C19

CYP2C19/S-mephenytoin 4’-hydroxylase – maximal activity found at 50 mM potassium phosphate, pH 7.4

CYP3A4/testosterone 6ß-hydroxylase – maximal activity found at 100-150 mM potassium phosphate, pH 7.4
“Preferably no more than 10-30% substrate or inhibitor depletion should occur. However, with low Km substrates, it may be difficult to avoid > 10% substrate depletion at low substrate concentrations.”

Midazolam is an example where short incubation times (≤ 5 min) and low protein concentrations (≤ 0.05 mg/mL) should be used to avoid excessive depletion

Rapidly metabolized substrates have the added benefits of:
- Reducing NCE depletion (that can lead to artifacts)
- Improved sensitivity to detect time-dependent inhibitors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Metabolite</th>
<th>Protein concentration (mg/mL)</th>
<th>Incubation time</th>
<th>Substrate concentration used for IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>0.2</td>
<td>10 min</td>
<td>40 µM</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Hydroxybupropion</td>
<td>0.1</td>
<td>5 min</td>
<td>80 µM</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>Desethylamodiaquine</td>
<td>0.02</td>
<td>5 min</td>
<td>1.5 µM</td>
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<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>4’-hydroxydiclofenac</td>
<td>0.05</td>
<td>5 min</td>
<td>5.0 µM</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin</td>
<td>4’-hydroxymephenytoin</td>
<td>0.3</td>
<td>10 min</td>
<td>40 µM</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Dextrorphan</td>
<td>0.1</td>
<td>5 min</td>
<td>5.0 µM</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>1’-hydroxymidazolam</td>
<td>0.02</td>
<td>5 min</td>
<td>3.0 µM</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>6ß-hydroxytestosterone</td>
<td>0.05</td>
<td>10 min</td>
<td>50 µM</td>
</tr>
</tbody>
</table>
## Validated LC/MS analytical methods

- Validated analytical methods should be used\(^1\)
- Should comply with USFDA’s Guidance for Industry-Bioanalytical Method Validation, May 2001
- High sensitivity LC/MS methods:
  - Permit use of low protein concentrations
  - Eliminates assay interference by NCE
  - Coupled with stable-isotope internal standards, ion suppression is negligible

### Parameters validated:
- Selectivity, standard curve and QC sample accuracy and precision, carryover, stability, autosampler stability, LLOQ
- Matrix: 0.1 mg/mL HLM, NADPH regenerating system
- >95% inhibition results in metabolite formation above the LLOQ

### Table: Standard Curve Range and Interday Precision

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Metabolite</th>
<th>Mass Transition</th>
<th>Internal Standard</th>
<th>Mass Transition</th>
<th>Ionization</th>
<th>LLOQ (µM) RE</th>
<th>CV</th>
<th>Range</th>
<th>( R^2 )</th>
<th>RE</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>151→111</td>
<td>Acetaminophen-([^{13}C_2]^{15}N]</td>
<td>155→110</td>
<td>ESI+</td>
<td>0.0780</td>
<td>108</td>
<td>11.9</td>
<td>0.076-5.0</td>
<td>0.9988</td>
<td>98-101</td>
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<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>4'-OH Diclofenac</td>
<td>312→268</td>
<td>4'-OH Diclofenac-([^{13}C_6]]</td>
<td>316→272</td>
<td>ESI+</td>
<td>0.0087</td>
<td>107</td>
<td>4.20</td>
<td>0.0087-2.0</td>
<td>0.9998</td>
<td>99-102</td>
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<tr>
<td>CYP2C19</td>
<td>S-mephenytoin</td>
<td>4'-OH S-Mephenytoin</td>
<td>235→150</td>
<td>4'-OH S-Mephenytoin-[D3]</td>
<td>238→150</td>
<td>ESI+</td>
<td>0.0040</td>
<td>101</td>
<td>10.4</td>
<td>0.004-10.0</td>
<td>0.9979</td>
<td>97-103</td>
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<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Dextrophan</td>
<td>258→157</td>
<td>Dextrophan-[D3]</td>
<td>261→157</td>
<td>ESI+</td>
<td>0.0025</td>
<td>94</td>
<td>7.90</td>
<td>0.0025-1.0</td>
<td>0.9977</td>
<td>94-103</td>
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<td>CYP3A4</td>
<td>Midazolam</td>
<td>1'-OH Midazolam</td>
<td>342→203</td>
<td>1'-OH Midazolam-([^{13}C_3]]</td>
<td>347→208</td>
<td>ESI+</td>
<td>0.0025</td>
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<td>0.0025-1.0</td>
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<td>6β-OH Testosterone</td>
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<td>6β-OH Testosterone-[D7]</td>
<td>312→276</td>
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<td>107</td>
<td>5.3</td>
<td>0.016-10.0</td>
<td>0.9994</td>
<td>98-102</td>
</tr>
</tbody>
</table>

\(^1\) – Specific statements are not made in the guidance but found later in Strong and Huang (2008)
Design Considerations – use of organic solvents

• “Any solvents should be used at low concentrations (< 1% (v/v) and preferably < 0.1%).

• Solvent effect can be substrate dependent

• Inhibition or, less commonly, activation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Solvent</th>
<th>0.1%</th>
<th>0.3%</th>
<th>1%</th>
<th>3%</th>
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<td>CYP2D6</td>
<td>Methanol</td>
<td>-</td>
<td>7</td>
<td>26</td>
<td>57</td>
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<tr>
<td></td>
<td>Ethanol</td>
<td>11</td>
<td>24</td>
<td>59</td>
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<td></td>
<td>DMSO</td>
<td>16</td>
<td>38</td>
<td>67</td>
<td>87</td>
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<tr>
<td></td>
<td>Acetonitrile</td>
<td>-</td>
<td>3</td>
<td>19</td>
<td>48</td>
</tr>
</tbody>
</table>

Design Considerations – positive control inhibitors

- “Use of an active control (known inhibitor) is optional.”
- Most laboratories use known inhibitors to demonstrate a properly functioning test system

Inhibition of HLM-catalyzed reactions by positive control reversible inhibitors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>IC50 (no preincubation)</th>
<th>K1 (no preincubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>7,8-Benzoflavone (ANF)</td>
<td>0.013 uM</td>
<td>0.019 uM</td>
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<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Ketoconazole</td>
<td>2.5 uM</td>
<td>0.98 uM</td>
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<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>Montelukast</td>
<td>0.026 uM</td>
<td>0.027 uM</td>
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<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>Sulfaphenazole</td>
<td>0.52 uM</td>
<td>0.20 uM</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin</td>
<td>Benzylninvanol</td>
<td>0.41 uM</td>
<td>0.34 uM</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Quinidine</td>
<td>0.62 uM</td>
<td>0.50 uM</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>Ketoconazole</td>
<td>0.016 uM</td>
<td>0.0089 uM</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>Ketoconazole</td>
<td>0.019 uM</td>
<td>0.021 uM</td>
</tr>
</tbody>
</table>

Results are means of duplicate determinations on independent days; Values were found to be within 2-fold of each other.
Current recommended approach to determine whether an NME is a reversible inhibitor

- "The likelihood of an in vivo interaction is projected based on the [I]/Ki ratio"
- "[I] represents the mean steady-state $C_{\text{max}}$ value for total drug (bound plus unbound) following administration of the highest proposed clinical dose"

<table>
<thead>
<tr>
<th>[I]/Ki</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>[I]/Ki &gt; 1</td>
<td>Likely</td>
</tr>
<tr>
<td>1 &gt; [I]/Ki &gt; 0.1</td>
<td>Possible</td>
</tr>
<tr>
<td>0.1 &gt; [I]/Ki</td>
<td>Remote</td>
</tr>
</tbody>
</table>

"An estimated [I]/Ki ratio of greater than 0.1 is considered positive and a follow-up in vivo evaluation is recommended."
Summary of Obach et al, 2006
Analysis of > 40 compounds with in vitro and in vivo DDI parameters
In vitro data able to identify those drugs that caused at least a 2-fold increase in the exposure to P450 marker substrate drugs
  - Except some mech. based inhibitors
If IC50 < 1 µM, DDI is likely
If IC50 > 10 µM (or even 50 µM), DDI is still possible
P450 in vitro inhibition data are valuable in designing clinical DDI study strategies
In vitro data can be used to predict the magnitudes of DDI

Determining Whether an NME is a Mechanism-Based Inhibitor

- “Time-dependent inhibition should be examined in standard in vitro screening protocols
- A 30-minute pre-incubation of a potential inhibitor before the addition of substrate is recommended.
- Any time-dependent and concentration-dependent loss of initial product formation rate indicates mechanism-based inhibition.
- Detection of time-dependent inhibition kinetics in vitro indicates follow-up with in vivo studies in humans.”

- This is a very active area of research
  - Heightened awareness of links between mechanism-based inhibitors, covalent binding and idiosyncratic toxicity
  - Surprisingly, little detail is provided in the Guidance document
- Recent approaches to in vivo predictions
Example Data – IC₅₀ Shifts

- Conditions:
  - Inhibition of CYP2C19 catalyzed S-mephenytoin 4’-hydroxylation in pooled HLM by rac-fluoxetine and isomers
  - 5-fold dilution of 30 minute preincubation with 1.5 mg/mL HLM and NADPH into secondary inc containing S-meph at K_M

- Comparison to 30 minute preincubation without NADPH

- (S) > Rac > (R)

- Fold shift for (S) found to be 28 ± 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ - NADPH</th>
<th>IC₅₀ + NADPH</th>
<th>IC₅₀ shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>(±)-fluoxetine</td>
<td>22, 37, 22</td>
<td>3.3, 3.9, 1.8</td>
<td>6.6, 9.4, 12</td>
</tr>
<tr>
<td>(S)-fluoxetine</td>
<td>91, 79, 109</td>
<td>2.8, 3.3, 4.0</td>
<td>33, 24, 28</td>
</tr>
<tr>
<td>(R)-fluoxetine</td>
<td>20, 22, 21</td>
<td>3.5, 3.9, 4.7</td>
<td>5.6, 5.5, 4.4</td>
</tr>
<tr>
<td>(S)-benzylnirvanol</td>
<td>0.19, 0.16, 0.17</td>
<td>0.41, 0.43, 0.44</td>
<td>0.46, 0.38, 0.38</td>
</tr>
<tr>
<td>ticlopidine</td>
<td>1.14, 1.20, 1.43</td>
<td>0.81, 0.76, 0.59</td>
<td>1.4, 1.6, 2.4</td>
</tr>
</tbody>
</table>

Each value represents a determination conducted on independent days. Global interday CV for all IC₅₀ values was 0.17. Values were not corrected for F_u.

Determination of $k_{\text{inact}}$ and $K_i$

- $k_{\text{inact}}$ and $K_i$ determinations are often conducted as a follow up to detection of TDI
  - Preincubation of multiple concentrations of NCE at multiple time point with NADPH

Recommendations for robust analysis:

- 10-fold dilution of the test article into a secondary incubation for $K_i/k_{\text{inact}}$ assays.
  - Minimizes the reversible/irreversible inhibition occurring in the secondary incubation.

- Substrate concentration should be ≥ 5X the Km
  - Further reduces reversible, competitive inhibition
  - Increases the velocity of the probe substrate reaction that can improve the analytical sensitivity.

- Number and spacing of preincubation times should be appropriate to accurately determine slope.
A dual time point IC50 shift strategy to optimize Kᵢ/kₖᵢₙₐct study design

Inhibition of Midazolam 1’-hydroxylase by selected CYP3A inhibitors

IC₅₀ Shift plot showing essentially no change in IC₅₀ shift after a 10 or 30 min preincubation demonstrates rapid inactivation

Conversely, IC₅₀ shift plots for verapamil and diltiazem show a significant shift between 10 and 30 min preincubation time points. This demonstrates verapamil and diltiazem are slow inactivators by comparison to azamulin.

**Kᵢ/kᵢₜₐₜ** determination with CYP3A4 in HLM

- Rapid inactivation by CYP3A4-catalyzed 1’-hydroxylation of midazolam by azamulin – close spacing of early time points
- Verapamil and diltiazem – slower inactivators
Relationship between shifted $IC_{50}$ and $K_i/k_{inact}$

- Good correlation between “shifted” $IC_{50}$ and $K_i$
- An even better correlation between shifted $IC_{50}$ and $k_{inact}/K_i$
- Assists in selection of NCE concentrations to test for $k_{inact}/K_i$ based solely on shifted IC50

Data from Obach et al (2007) Drug Metab Dispos 35:246-255
When will the draft guidance be finalized?

- Only the FDA knows…
  - Go to: Clinical Pharmacology

**Clinical Pharmacology**

<table>
<thead>
<tr>
<th>Title and Format</th>
<th>Type</th>
<th>Issue Date</th>
</tr>
</thead>
</table>
| Clinical Lactation Studies--Study Design, Data Analysis, and Recommendations for Labeling [HTML] or [PDF] | Draft | Issued 2/7/05
| Federal Register Notice [TXT] [PDF]                                             | note* |             |
|                                                                                 | note* |             |
Can we expect significant changes in the draft?

- Only the FDA knows…
- > 25 comments have been submitted [http://www.fda.gov/ohrms/dockets/dockets/06d0344/06d0344.htm](http://www.fda.gov/ohrms/dockets/dockets/06d0344/06d0344.htm)
- PhRMA has submitted comments representing several pharma
Some sample comments

• “We believe that all drugs under development should be subjected to a cocktail study to elucidate whether an in vivo drug interaction occurs with the major CYP enzymes, regardless of the in vitro findings”

• Page 33 Line 1025, “Use of an active control (known inhibitor) is optional.”

  The use of an active control is appropriate for all experiments.

• Page 34 Lines 1065 – 1074, “Time-dependent inhibition should be examined in standard in vitro screening protocols, because the phenomenon cannot be predicted with complete confidence from chemical structure. A 30-minute pre-incubation of a potential inhibitor before the addition of substrate is recommended. Any time-dependent and concentration-dependent loss of initial product formation rate indicates mechanism-based inhibition. For compounds containing amines, metabolic intermediate complex formation can be followed spectrascopically. Detection of time-dependent inhibition kinetics in vitro indicates follow-up with in vivo studies in humans.”

  The mechanism-based inhibitor section is not well developed. Additional content may help better explain this section.
Recommended strategies for robust analysis

- Follow recommendations in the draft FDA guidance
- Until document is final, consider the docket comments
- Consider “consensus” documents published by representatives from industry and/or government
- GLP is not required
  - Laboratories conducting these assays should be GLP compliant
  - Validated methods, Data tracking processes, SOPs, etc
- Use well accepted reagents from reputable suppliers
- Use microsomes as enzyme source
  - Lack of confounding cellular processes vs hepatocytes, slices
  - More practical (e.g. large, long-lasting HLM pools; renewable cDNA-expressed)
Recommended strategies for robust analysis

• Direct inhibition assessment:
  – Determine IC50 with 7 CYPs
  – 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4
  – Use two substrates for 3A4
  – Follow-up Ki with ≥ 5 substrate concentrations

• Examine the potential for mechanism-based inhibition
  – Preincubation of test article with NADPH
  – IC50 shift – preliminary test for time-dependent inhibition
  – If > 2-fold shift, determine $K_i$ and $k_{\text{inact}}$

• Integrate findings with other data points
  – Free fraction in plasma, microsomes
  – Substrate of transporters?
  – Patient population, route of administration, nonCYP metabolism
• Provides GLP and non-GLP CYP inhibition testing services.
• Provides key reagents:
  – Highly characterized pooled HLM and rCYP
  – Model inhibitors, substrates & metabolites (including heavy labeled)
  – Buffers, cofactors and associated Labware
Questions?

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David_Stresser@bd.com
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