New regulatory & experimental developments in the *in vitro* approaches to assess the victim & perpetrator potential of drug candidates

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**Victims (objects) versus Perpetrators (precipitants)**

<table>
<thead>
<tr>
<th>Victims</th>
<th>Perpetrators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearance determined by a single route of elimination</td>
<td>Factors that alter the clearance of a victim drug</td>
</tr>
</tbody>
</table>
| **Victim drugs – Not approved**  
Debrisoquine, perhexiline | **Genetic polymorphisms**  
CYP2D6 (PMs and EMs) |
| **Victim drugs – Withdrawn**  
Terfenadine, cisapride, astemizole and cerivastatin | **Inhibitory drugs**  
Erythromycin, ketoconazole, Mibefradil (withdrawn) |
| **Victim drugs – Loss of efficacy**  
Oral contraceptive steroids, HIV and immunosuppressive drugs | **Inducing drugs**  
Rifampin, EIAEDs, SJW  
None withdrawn, but may be denied approval (AIDS patients) |
Infomercial presented at the JSSX meeting in Kumamoto

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FDA guidance on in vitro ADME studies to evaluate the victim and perpetrator potential of drug candidates (draft issued in 2006)

Emphasis before 2006
Six CYP enzymes: 1A2, 2C8, 2C9, 2C19, 2D6 and 3A4

Emphasis after 2006
Seven CYP enzymes: 1A2, 2C8, 2C9, 2C19, 2D6, 3A4 and 2B6

The eighth CYP enzyme? I predict CYP2J2
Specific marker in HLM = ebastine hydroxylation

Transporters (2006):
Emphasis on P-glycoprotein
Other transporters (e.g., OATP's)
Other CYP enzymes (e.g., CYP2J2)
Conjugation (UGT, SULT)
Oxidation (by non-CYP enzymes)
Reduction and hydrolysis

Presentation overview

Some new trends and unexpected findings from in vitro ADME studies:

Oxidation and reduction
Unusual CYP enzymes
Aldehyde oxidase and cytochrome b5

Glucuronidation
Underestimation of clearance
Problems with inhibition studies

CYP inhibition
Metabolism-dependent inhibition: Role of non-CYP enzymes
An ultracentrifugation method to study MDI mechanisms
CYP inhibition in the presence of human plasma

Final slide has information on how to obtain a copy of this presentation
CYP reaction phenotyping, the usual CYP suspects have expanded, but other CYP enzymes may be important

The FDA's big six is now the big seven

CYP1A2, 2C8, 2C9, 2C19, 2D6, 3A4 and **CYP2B6**

However, some drug candidates are metabolized by other CYP enzymes

**Example:** DB285 is mainly metabolized by CYP4F enzymes

These are rarely considered in reaction phenotyping studies

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The pro-drug DB289 is metabolized by CYP4F2 and 4F3b

![Chemical structure of DB289 and Amidoxime]

O-Demethylation by CYP4F2 and CYP4F3b with some contribution from CYP2J2

Cytochrome b₅ and aldehyde oxidase can catalyze certain dehydroxylation reactions

The microsomal hemoprotein cytochrome b₅ and the cytosolic molybdozyme aldehyde oxidase can both catalyze certain dehydroxylation reactions.
Presentation overview

Some new trends and unexpected findings from *in vitro* ADME studies:

**Oxidation and reduction**
- Unusual CYP enzymes
- Aldehyde oxidase and cytochrome b5

**Glucuronidation**
- Underestimation of clearance
- Problems with inhibition studies

**CYP inhibition**
- Metabolism-dependent inhibition: Role of non-CYP enzymes
- An ultracentrifugation method to study MDI mechanisms
- CYP inhibition in the presence of human plasma

Rates of microsomal glucuronidation *in vitro* seriously underestimate *in vivo* rates of glucuronidation

Alamethicin increases Vmax for AZT glucuronidation

![Graph showing the increase in AZT glucuronidation](image)

Yerino et al. JSSX Abstract 417 (Sendai, Japan, 2008)
BSA (but not HSA) decreases Km for AZT glucuronidation

Yerino et al. JSSX Abstract 417 (Sendai, Japan, 2008)

Rates of microsomal glucuronidation \textit{in vitro} seriously underestimate \textit{in vivo} rates of glucuronidation

Ala + BSA increases Vmax/Km for AZT glucuronidation \~14 fold

Yerino et al. JSSX Abstract 417 (Sendai, Japan, 2008)
Ala + BSA is good for UGT2B7 but bad for all UGT1A enzymes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>UGT</th>
<th>Vmax / Km</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Native</td>
</tr>
<tr>
<td>AZT</td>
<td>UGT2B7</td>
<td>1.3</td>
</tr>
<tr>
<td>Morphine</td>
<td>UGT2B7</td>
<td>13</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>UGT1A1</td>
<td>51</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>UGT1A4</td>
<td>31</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>UGT1A6</td>
<td>6880</td>
</tr>
<tr>
<td>Propofol</td>
<td>UGT1A9</td>
<td>188</td>
</tr>
</tbody>
</table>

Yerino et al. JSSX Abstract 417 (Sendai, Japan, 2008)

UGT – Problems with inhibition studies

The ability of rapidly conjugated substrates to inhibit UGT activities can differ between human liver microsomes and recombinant enzymes, and the degree of inhibition is influenced by the concentration of the cofactor UDP-glucuronic acid (UDPGA).

There can be non-competitive metabolism-dependent inhibition of UGT enzymes due to depletion of UDPGA and formation of UDP.

There can be metabolism-dependent protection of UGT enzymes due to conjugation of the inhibitor.

This impacts the design of in vitro UGT inhibition assays.
The inhibition of UGT1A4 and UGT1A9 by 1-naphthol (a high turnover UGT1A6 substrate) depends on the UGT source (HLM versus rUGT)

Degree of inhibition of UGT1A4 (trifluoperazine): HLM > rUGT
Degree of inhibition of UGT1A9 (propofol)): UGT > HLM

\[ \text{UDPGLA} = 0.2 \text{ mM} \]

The inhibition of UGT1A4 and UGT1A9 by 1-naphthol (a high turnover UGT1A6 substrate) depends on the concentration of UDPGA (cofactor)

Degree of inhibition is less at 20 mM UDPGA compared with 0.2 mM UDPGA

Enzyme source: Human liver microsomes
UGT – Problems with inhibition studies

Why?
Conjugation of 1-naphthol by UGT1A6 decreases its inhibitory effect on UGT1A9. Such metabolism-dependent protection occurs with HLM but not with recombinant UGT1A9.

Rapid conjugation of 1-naphthol by UGT1A6 has two effects:
- It decreases UDPGA (= loss of cofactor)
- It increases UDP (= formation of competitive inhibitor of UDPGA)

Such non-competitive metabolism-dependent inhibition occurs with HLM but not with recombinant UGT1A4.

Fujiwara, Nakajima, Yamanaka, Katoh and Yokoi
Product inhibition of UDP-glucuronosyltransferase enzymes by UDP obfuscates the inhibitory effects of UGT substrates
*Drug Metab. Dispos.* **36**: 361-367, 2008

Presentation overview

Some new trends and unexpected findings from *in vitro* ADME studies:
- Oxidation and reduction
  - Unusual CYP enzymes
  - Aldehyde oxidase and cytochrome b5
- Glucuronidation
  - Underestimation of clearance
  - Problems with inhibition studies

CYP inhibition
- Metabolism-dependent inhibition: Role of non-CYP enzymes
- An ultracentrifugation method to study MDI mechanisms
- CYP inhibition in the presence of human plasma
Metabolism-dependent inhibition of CYP enzymes may involve metabolism by enzymes other than cytochrome P450

Example A: Inhibition of CYP2C8 by gemfibrozil – role of glucuronidation

Example B: Inhibition of CYP2D6 by bupropion – role of carbonyl reduction

Cerivastatin was withdrawn from the US, European and Japanese markets because of adverse and sometimes fatal side effects (rhabdomyolysis)

One third of fatal cases of rhabdomyolysis involved coadministration of cerivastatin with the lipid-lowering drug gemfibrozil

Cerivastatin is cleared by CYP2C8 and, to a lesser extent, CYP3A4

However, gemfibrozil is NOT a potent inhibitor of CYP2C8 or 3A4

How does gemfibrozil inhibit the metabolism of cerivastatin?
Role of glucuronidation in the inhibition of CYP2C8 by gemfibrozil

Gemfibrozil is extensively glucuronidated

Gemfibrozil glucuronide - but NOT gemfibrozil itself - is a mechanism-based inhibitor of CYP2C8

This explains why gemfibrozil blocks the metabolism of cerivastatin and increases its toxicity

Gemfibrozil and its glucuronide metabolite can inhibit CYP2C8

Mechanism-based inhibition of CYP2C8


Clinical evidence that gemfibrozil is a metabolism-dependent inhibitor of CYP2C8 (with repaglinide as the probe substrate) and also an inhibitor of OATP1B1

Tornio A, Niemi M, Neuvonen M, Laitila J, Kalliokoski A, Neuvonen PJ and Backman JT.

The effect of gemfibrozil on repaglinide pharmacokinetics persists for at least 12 h after the dose: Evidence for mechanism-based inhibition of CYP2C8 in vivo.

Clin Pharmacol Ther. 84: 403-411, 2008
Role of non-CYP metabolism in CYP2D6 inhibition by bupropion
Clinically relevant interaction between desipramine (victim) and bupropion (perpetrator)

Reese MJ, Wurm RM, Muir KT, Generaux GT, Si John-Williams L, and McConn DJ.
An in vitro mechanistic study to elucidate the desipramine/bupropion clinical drug-drug interaction.

Role of non-CYP metabolism in CYP2D6 inhibition by bupropion
Oxidative and reductive metabolism of bupropion

Xu, H et al., Stereoselective analysis of hydroxybupropion and application to drug interaction studies.

Bondarev, M. Behavioral and biochemical investigations of bupropion metabolites
Role of non-CYP metabolism in CYP2D6 inhibition by bupropion

*In vitro* inhibition of CYP2D6 by bupropion

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Estimated human liver concentration (µM)</th>
<th>Ki (µM)</th>
<th>Median predicted AUC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bupropion</td>
<td>0.61 – 1.0</td>
<td>21</td>
<td>1.03</td>
</tr>
<tr>
<td>Hydroxybupropion</td>
<td>5.1 – 8.7</td>
<td>13</td>
<td>1.44</td>
</tr>
<tr>
<td>Threohydrobupropion</td>
<td>7.4 – 13</td>
<td>5.4</td>
<td>2.40</td>
</tr>
<tr>
<td>Erythrohydrobupropion</td>
<td>1.4 – 2.4</td>
<td>1.7</td>
<td>1.88</td>
</tr>
<tr>
<td><strong>Combined</strong></td>
<td><strong>3.31</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ultracentrifugation as an alternative to dialysis and dilution as a method to investigate the mechanism of MDI

Test article (or positive control) is preincubated (zero and 30 min) with a low concentration of HLM (≤ 0.1 mg/mL) ± NADPH (to distinguish TDI from MDI)

Samples divided into 4 groups:

- **Sample 1**: Incubated with solvent (negative control) but not centrifuged
- **Sample 2**: Incubated with TA (or positive control) but not centrifuged
- **Sample 3**: Incubated with TA (or positive control) and then centrifuged
- **Sample 4**: Incubated with TA (or positive control) and then treated with 2 mM K$_3$Fe(CN)$_6$ (to dissociate quasi-irreversible MI complexes) prior to centrifugation,

The centrifuged samples are resuspended prior to determining CYP activity
Examples of the ultracentrifugation method
Irreversible inhibition of CYP3A4 by mibefradil (2 µM), an irreversible MDI of CYP3A4

![Graph showing testosterone 6β-hydroxylation](image1)

Examples of the ultracentrifugation method
Irreversible inhibition of CYP3A4 by troleandomycin (25 µM), a quasi-irreversible MDI of CYP3A4 (forms an MI complex)

![Graph showing testosterone 6β-hydroxylation](image2)
Advantages of the ultracentrifugation method

- The test article is incubated with a low concentration of microsomal protein (not a 10- to 25-fold higher concentration as is the case with the dilution method and, generally, the dialysis method)

- This avoids problems associated with the use of a higher protein concentration (such as incomplete enzyme inactivation due to a reduction in the free concentration of inhibitor or over-metabolism of the inhibitor)

- Avoids a potentially problematic and lengthy dialysis procedure

- Discriminates certain types of quasi-irreversible from irreversible inhibition based on reversibility by potassium ferricyanide

  Certain types of quasi-irreversible inhibition can be reversed by oxidizing the heme ferrous iron to the ferric state with potassium ferricyanide, which dissociates the MI complex

Adding human plasma to *in vitro* incubation systems

We have investigated whether it is possible to conduct inhibition studies with HLM and hepatocytes in the presence of human plasma

If they can, the *in vivo* concentration of free drug in plasma will be the same as the *in vitro* concentration of free drug (because it’s also in plasma).

IVIVE can then be **correctly** based on total drug concentration (Cmaxss) as is required by the FDA, without even knowing what the free concentration of drug is (because it’s the same *in vitro* and *in vivo*).

Plasma would be expected to change Km for marker substrates and Ki for inhibitors for those drugs that bind extensively to plasma protein (e.g., acidic drugs)
Effect of human plasma on CYP3A4 inhibition in HLM
Ritonavir (versus midazolam)

**Experimental Conditions**
- HLM at 0.05 mg/mL
- ~95% Human plasma
- 5 min incubation with midazolam
- $[S] = 4 \mu M$ (Km in buffer)

**IVIVE**
- Without plasma: predict CYP3A4 inhibition
  - Correct answer: Inhibition
- With plasma: predict CYP3A4 inhibition

IC\textsubscript{50}\textsubscript{Buffer} = 0.026 \mu M
IC\textsubscript{50}\textsubscript{Plasma} = 0.35 \mu M

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Effect of human plasma on CYP2C8 inhibition in HLM
Montelukast

**Experimental Conditions**
- HLM at 0.1 mg/mL
- ~95% Human plasma
- 5 min incubation with amodiaquine
- $[S] = 7 \mu M$ (Km buffer)

**IVIVE**
- Without plasma: predict CYP2C8 inhibition
  - Correct answer: Inhibition
- With plasma: predict no CYP2C8 inhibition

IC\textsubscript{50}\textsubscript{Buffer} = 0.47 \mu M
IC\textsubscript{50}\textsubscript{Plasma} = 60 \mu M
Effect of human plasma on CYP2D6 inhibition in HLM

Terbinafine

**Experimental Conditions**
- HLM at 0.1 mg/mL
- ~95% Human plasma
- 5 min incubation with dextromethorphan
- \([S] = 7.5 \mu M\) (Km buffer)

**IVIVE**
- Without plasma: predict CYP2D6 inhibition
- With plasma: predict no CYP2D6 inhibition
- Correct answer: Inhibition

**Effect of adding plasma to HLM on IVIVE for CYP inhibition**

<table>
<thead>
<tr>
<th>CYP enzyme</th>
<th>Inhibitor</th>
<th>Correct answer obtained with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>Ketoconazole</td>
<td>Both plasma and buffer</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>Both plasma and buffer</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Quinidine</td>
<td>Both plasma and buffer</td>
</tr>
<tr>
<td></td>
<td>Terbinafine</td>
<td>Buffer (plasma under-predicted)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Montelukast</td>
<td>Plasma (buffer over-predicted)</td>
</tr>
</tbody>
</table>

**Conclusions**
- CYP inhibition studies with HLM can be performed in the presence of 96% human plasma, which achieves the same *in vitro* concentration of free drug as that present *in vivo*
- Plasma greatly affects substrate Km and inhibitor Ki especially for acidic drugs
- Does adding plasma improve IVIVE? In some cases YES, in other cases NO
References

Chapter 6
A. Parkinson and B.W. Ogilvie
Xenobiotic biotransformation.
Chapter 6 in: Casarett and Doull’s Toxicology. The Basic Science of Poisons.

Chapter 7
B.W. Ogilvie, E. Usuki, P. Yerino and A. Parkinson.
In vitro approaches for studying the inhibition of drug-metabolizing enzymes and
identifying the drug-metabolizing enzymes responsible for the metabolism of
drugs (reaction phenotyping) with emphasis on cytochrome P450.
Chapter 7 in: Drug-Drug Interactions (Drugs and the Pharmaceutical Sciences). Second

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Domo Arigato
Gozaimasu

With special thanks
to
Sekisui Medical

For a copy of this presentation, please go to:
www.sekisuimedical.jp/business/adme_tox/index.html

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