CYP Induction Assays in Plated Hepatocytes: CYP Activity and mRNA Analysis

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BD Biosciences
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Presentation Overview

• Induction Protocol and Assay Methods
• Experimental design for P450 induction studies
  – Choice of model
  – Choice of enzymes
  – Incubation period
  – Positive control inducers
  – End points
  – Data Interpretation
• Other technical considerations
• Activity vs. mRNA assays
Why Is Enzyme Induction a Concern for Drug Candidate Developability?

- Therapeutic failure and safety issues
  - Higher rate of drug inactivation, so less of the (oral) parent drug reaches target
    - Autoinduction (self)
    - Drug-interaction (co-medication)
  - More potentially toxic metabolite
- May be clinically manageable
  - Reduction in drug concentration can be circumvented by increasing drug dosage
- Regulatory agencies expects data for enzyme induction potential

Wilkinson GR N Engl J Med 352;2211, 200
Examples-Clinical Drug Interactions Due to Induction

- Co-medication of rifampicin with warfarin reduces plasma concentration of warfarin, leading to a reduction in the thrombin time.
- Co-medication of rifampicin with oral contraceptives resulted in unplanned pregnancies.
- Co-medication of rifampicin with rosiglitazone caused 60% decrease in AUC, Cmax, and T½ of rosiglitazone.
- Therapeutic failure due to co-medication of cyclosporine or HIV-protease inhibitors with St. John’s wort.
Regulatory Guidance For DDI

- EMA (April, 2010) DRAFT Guideline on the Investigation of Drug Interactions
Protocol for Induction Studies

Hepatocytes are plated in multiwell plates or dishes (1-3 days)

2-3-day treatments with test article and positive control inducers

- mRNA
- Enzyme activity “in situ”
- Adjunct RT-PCR or Western blot with cells
- Harvest cells and prepare microsomes
- Microsomal enzyme activity
  Western blot w/ microsomal protein
## Analytical-API4000 LC/MS Platform

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CYTP1A2</th>
<th>CYTP2B6</th>
<th>CYTP3A4</th>
<th>CYTP2C9</th>
<th>CYTP2C8</th>
<th>CYTP2C9</th>
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</thead>
<tbody>
<tr>
<td>Substrate (µM)</td>
<td>Phenacetin (100)</td>
<td>Bupropion (250)</td>
<td>Testosterone (200)</td>
<td>Diclofenac (100)</td>
<td>Amodiaquine (100)</td>
<td>S-Mephenytoin (200)</td>
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<tr>
<td>Metabolite</td>
<td>Acetamidophenol</td>
<td>Hydroxybupropion</td>
<td>6β-Hydroxytestosterone</td>
<td>4'-Hydroxydiclofenac</td>
<td>Desethylamodiaquine</td>
<td>4'-Hydroxy-S-mephenytoin</td>
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<td>Incubation Time (min)</td>
<td>60</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Standard curve range (µM)</td>
<td>0.076-5</td>
<td>0.000488-0.8</td>
<td>0.016-10</td>
<td>0.00867-2</td>
<td>0.00469-1.5</td>
<td>0.0039-10</td>
</tr>
<tr>
<td>Internal Standard (µM)</td>
<td>Acetamidophenol-[^13C2][^15N] (10)</td>
<td>Hydroxybupropion-[^D6] (0.1)</td>
<td>6β-Hydroxytestosterone-[^D7] (5)</td>
<td>4'-Hydroxy diclofenac-[^13C6] (0.5)</td>
<td>Desethylamodiaquine-[^D3] (0.1)</td>
<td>4'-Hydroxy S-Mephenytoin-[^D3] (0.5)</td>
</tr>
<tr>
<td>Ionization Mode</td>
<td>ESI+</td>
<td>ESI+</td>
<td>ESI+</td>
<td>ESI+</td>
<td>ESI+</td>
<td>ESI+</td>
</tr>
<tr>
<td>Mass Transitions (Analyte)</td>
<td>152→110</td>
<td>256→139</td>
<td>305→269</td>
<td>312→268</td>
<td>330→285</td>
<td>235→150</td>
</tr>
<tr>
<td>Mass Transitions (Internal)</td>
<td>155→111</td>
<td>262→244</td>
<td>312→278</td>
<td>316→272</td>
<td>333→285</td>
<td>238→150</td>
</tr>
</tbody>
</table>
Real-time RT-PCR Analysis

• RNA Isolation
  – Trizol or RNeasy 96 kits

• One-step Real-time RT-PCR
  – Required reagents:
    • Template DNA (cDNA from reverse transcription)-isolated total RNA from hepatocytes
    • MultiScribe reverse transcriptase
    • Primers
    • TaqMan probes (fluorogenic with fluorescent quencher)
    • DNA polymerase (AmpliTaq DNA polymerase)
    • dNTP
  – Follow the gene expression kit protocol
  – Determine PCR amplification efficiency
  – Applied Biosystems 7300/7500/7900 Real-Time PCR System
Fold induction = $2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT_{\text{control}} - \Delta CT_{\text{induced}}$
Experimental Design—Choice of Model

- **Primary cultures of animal hepatocytes**
  - Use of animal hepatocytes for induction studies is not recommended for predicting human response.

- **Immortalized hepatic cell lines such as HepG2, HepaRG, and Fa2N-4**
  - FDA-“Immortalized liver cells are acceptable if it can be demonstrated with positive controls that CYP3A4 and CYP1A2 are inducible in these cell lines.”
  - EMA-“Well-validated cell lines with proven inducibility via the regulation pathway may be used. Has to be scientifically very well justified.”

- **Primary cultures of human hepatocytes (fresh/cryopreserved)—gold standard**
  - FDA-“Experiments should be conducted with hepatocytes prepared from at least three individual donor livers.”
  - EMA-“It is recommended to use hepatocytes from at least 3 different donors.”
Inter-Individual Variation- Example of CYP3A4 Induction by RIF in 64 Donors

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>RIF</th>
<th>Fold induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Max</td>
<td>567</td>
<td>2171</td>
<td>122</td>
</tr>
<tr>
<td>Min</td>
<td>6.5</td>
<td>239</td>
<td>1.4</td>
</tr>
<tr>
<td>Median</td>
<td>38</td>
<td>930</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>83</td>
<td>957</td>
<td>28</td>
</tr>
</tbody>
</table>
Experimental Design- Choice of Enzymes

• **FDA** “The initial in vitro induction evaluation may include only CYP1A2 and CYP3A4.”
  – Although CYP2B6, 2C8, 2C9 and 2C19 are inducible, they co-induce with CYP3A4, which can serve as a surrogate. However, many investigators like to include these enzyme to avoid the need to retest, to examine inducers acting via CAR or circumvent 3A4-selective inhibitors.

  – Update: “Because CYP2B6 may not be co-induced with CYP3A4, the potential for induction of CYP2B6 should be evaluated regardless of the CYP3A results”- Huang, S-M. J. Clin. Pharmacol. 48:662 (2008)

• **EMA** “The enzymes CYP3A4, CYP2B6 and CYP1A2 should always be included”
Example—Co-Induction of CYP2B6 and CYP3A4

- 31 New Chemical Entities (NCEs) were analyzed for CYP2B6 and CYP3A4 induction (mRNA)
- Close concordance of CYP2B6 induction with CYP3A4 induction found
- However, there were some notable exceptions
- Some compounds found to be 2B6 inducers in the absence of significant 3A4 induction; these may be acting as preferential CAR activators
- Look for induction of CYP2B6 in final FDA guidance document
Concentration of positive control inducers should be used at which a maximal induction response is obtained. This is critical since this will affect response of % of positive control, which is used for prediction of enzyme induction.

<table>
<thead>
<tr>
<th>CYP</th>
<th>FDA</th>
<th>EMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>25-100 μM omeprazole; 33-50 μM β-naphthoflavone; 1-2 μM 3-methylcholanthrene</td>
<td>25-50 μM omeprazole</td>
</tr>
<tr>
<td>2B6</td>
<td>500-1000 μM phenobarbital</td>
<td>500-1000 μM phenobarbital; ≤0.1 μM CITCO</td>
</tr>
<tr>
<td>3A4</td>
<td>10 μM rifampicin</td>
<td>20 μM rifampicin</td>
</tr>
</tbody>
</table>
Example—Selection of Concentration of Positive Control Inducers-CYP1A2

Concentration-dependent induction of CYP1A2 by BNF

Zhang et al. Drug Metabolism Letters 4:185, 2010
Example—Selection of Concentration of Positive Control Inducers-CYP2B6

Concentration-dependent induction of CYP1A2 by PB

Zhang et al. Drug Metabolism Letters 4:185,2010
Example—Selection of Concentration of Positive Control Inducers-CYP3A4

Concentration-dependent induction of CYP3A4 by RIF

Zhang et al. Drug Metabolism Letters 4:185,2010
Experimental Design—Concentrations of Test Articles

FDA

• Based on the expected human plasma drug concentrations

• At least 3 concentrations spanning the therapeutic range including 1 concentration that is an order of magnitude > the average expected plasma drug concentration

• If information is not available, concentrations ranging over at least 2 orders of magnitude

EMA

• The investigational drug concentration range that needs to be investigated depends on enzyme studied, and the *in vivo* pharmacokinetics of the drug.

• The studied exposure range, i.e. range of average concentration in the media (Cavg), should cover the concentrations given “above”. Such as “upper concentration”-50-fold the unbound C\(\text{max}\) or 250-fold the unbound C\(\text{max}\) for drugs with a plasma protein binding >99%.
Exposure time should be used at which a robust induction should be achieved. A 2-3 day treatment is recommended in draft guidance.

Time-dependent induction of CYP1A2 by BNF

Zhang et al. Drug Metabolism Letters 4:185,2010
Experimental Design—Exposure Time for CYP2B6 Induction

Time-dependent induction of CYP2B6 by PB

Zhang et al. Drug Metabolism Letters 4:185,2010
Experimental Design—Exposure Time for CYP3A4 Induction

Time-dependent induction of CYP3A4 by RIF

Zhang et al. Drug Metabolism Letters 4:185,2010
Experimental Design—Choice of Endpoints

FDA

• The most reliable method to study a drug’s induction potential is to quantify the enzyme activity of (human) primary hepatocyte culture.
• Measurement of mRNA levels is helpful when both enzyme inhibition and induction are operative.”

EMA

• It is recommended to measure the extent of enzyme induction as enzyme activity.
• mRNA measurement is mandatory for the interpretation of study results if inhibition of the studied enzyme may not be excluded…or a down-regulation is suspected based on the activity assay.
• If both activity and mRNA are measured, activity results prevail unless enzyme inhibition is indicated.
How to Define an Inducer?

FDA

- “A drug that produces a change that is equal to or greater than 40% of the positive control can be considered as an enzyme inducer *in vitro* and *in vivo* evaluation is warranted.”
- “An alternative endpoint is the use of an EC50 (effective concentration at which 50% maximal induction occurs)”
- Both EC$_{50}$ and E$_{\text{max}}$ are important parameters for calculation of relative induction score (RIS).

EMA

- Negative results- below 1.5-fold induction or less than 20% of the response to positive control inducers.
Sensitivity: Activity vs. mRNA

CYP3A4 Induction (N=16)

CYP1A2 Induction (N=13)

CYP2B6 Induction (N=13)
Sensitivity: Activity vs. mRNA

McGinney et al. Drug Metabolism and Disposition 37:1259, 2009
Other Technical Considerations

- CYP inhibition/inactivation by the inducing agent or its metabolites ("masking")
- Test article solubility in medium
- Actual concentration of drug in the system; determine free concentration in the system and use it to calculate the parameters such as EC$_{50}$ or E$_{max}$ (EMA guideline)
- Activity of metabolite > parent
- PXR/CAR mediated induction of transporters/other enzymes
- Pharmacogenetic variation (use multiple donors)
- “Down-regulation”
- Cytotoxicity
Test for Masking of Enzyme Activity by NCE

• Enzyme activity assays are gold-standard for induction testing. However, enzyme activity may be inhibited by the NCE (“masking”) – the inhibition can be due to either direct or time-dependent inhibition.

• A direct inhibition test can be performed with a separate set of positive control inducer treated wells (in situ assay).
  – Ensures high starting activity

• The potential confounding effect of enzyme inhibition on enzyme induction response can be uncovered with mRNA or Western blot analysis
Inhibition assay: test item (in this case TAO or ritonavir) added at end of 3d treatment for 30 minutes, followed by wash and addition of probe substrate.
Hepatocytes from two donor livers were treated with TAO (0.2, 2, and 20 µM) for 3 days. After treatment, CYP3A4 activity and mRNA expression were determined.
Inducers with Time-Dependent Inhibitor

Fig. 1. Relationship between enzyme activity and mRNA $E_{\text{max}}$ values from HH205 and HH215. The $E_{\text{max}}$ values were determined in HH205 (A) and HH215 (B) using CYP3A4 activity and mRNA as the endpoints as outlined under Materials and Methods. Open circles, CYP3A4 time-dependent inhibitors as determined in HLMs; closed circles, compounds that were negative in this assay. All compounds with a mRNA/activity ratio of $>10$ are labeled.
Apparent Down-Regulation in Enzyme Activity and mRNA

- CYP3A4 enzyme activity demonstrates apparent down-regulation
- mRNA supports this conclusion
- No inhibition of enzyme activity found in liver microsomes or in hepatocytes in situ
- No hepatocyte toxicity was found
Recommended Strategies for Robust Analysis

- Follow recommendations in the draft Regulatory guidance
- 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 human hepatocyte for induction for late discovery and/or development drug candidates.
Key BD Gentest Products and Services for Induction Studies

- BD Gentest™ Fresh and Inducible Human Hepatocytes
- BD BioCoat™ Culture Plates and Flasks
- BD™ Hepatocyte Culture Medium
- BD Gentest CryoHepatocyte Purification Kit
- BD Gentest Heavy-Labeled Metabolite Standards
- Comprehensive induction services including mRNA in situ and microsomal assays. Hepatocyte toxicity assays are also available.
- New High Viability Recovery and Plating Media for Human Platable Cryopreserved Hepatocytes
Questions?

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