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# In Vitro Induction Studies: Elements of Design and Important Considerations in Data Analysis

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**Vice President of Scientific Operations**

# Overview

- Introduction
  - Enzyme induction basics
  - Relevance
- Standard study design
  - What is included and why?
- After initial data generation
- Options for next steps
- Unanswered questions
- Conclusions

# Enzyme induction basics

- Drugs can cause induction (up-regulation) of drug-metabolizing enzymes
  - E.g., Cytochrome P450 (CYP) enzymes
- Primary mechanism is activation of gene transcription
  - Often by nuclear receptor activation

| Enzyme | Receptor | Acronym |
|---|---|---|
| CYP1A2 | Aryl hydrocarbon receptor | AhR |
| CYP2B6 | Constitutive androstane receptor | CAR |
| CYP3A4 etc | Pregnane X receptor | PXR |

- PXR activation is the most common
  - Large / flexible binding pocket
- Species differences are prevalent

Drug Metab Dispos. 2009 Jul;37(7):1339-54

# Enzyme induction relevance

- Drug metabolizing enzyme induction can alter metabolic clearance
  - Of the inducer itself: auto-induction
  - Of concomitantly administered drugs
- This needs evaluating for drug-drug interaction (DDI) potential

- Efficacy of narrow therapeutic index drugs can be compromised
  - Various common antiepileptics (e.g., carbamazepine, phenobarbital, phenytoin) induce P450 enzymes and speed up clearance of comeds (e.g., warfarin), reducing exposure and so efficacy

Drug Metab Dispos. 2009 Jul;37(7):1339-54.
Brodie et al. *Epilesia* 2013, 54(1): 11-27.

# Guidance requirements

| Requirement | FDA (2017) | EMA (2013) |
|---|---|---|
| **Test system (number of donors)** | Cryopreserved or fresh human hepatocytes (*other systems considered complimentary*) minimum three donors | |
| **TA concentrations** | Expected or observed human plasma drug concentrations or intestinal drug concentrations (CYP3A4/5) and span therapeutic exposures and an order of magnitude above Cmax-ss unbound | 50x Cmax-ss unbound or if orally dosed 1/10$^{th}$ dose in 250 mL for CYP3A4 |
| **CYP emphasis** | 1A2, 2B6, 3A4 | 1A2, 2B6, 3A4 |
| **Controls** | Negative: Not specified<br><br>CYP specific Positive:<br>Omeprazole (25-100 µM)<br>Phenobarbital (500-1000 µM)<br>Rifampin (10-50 µM) | Negative: Not required<br><br>CYP specific Positive:<br>Omeprazole (50 µM)<br>CITCO (100 nM)<br>Rifampin (20 µM |

FDA 2017 Guidance for Industry: In vitro metabolism- and transporter-mediated drug-drug interaction studies.

EMA 2013 Guideline on the investigation of drug interactions

# A standard study

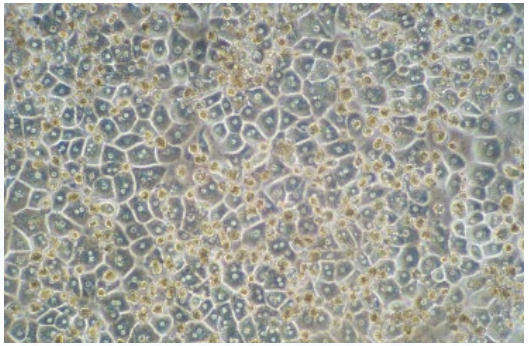
- Supporting / preliminary tests
- Cryopreserved cultured hepatocytes
  - 3 individual donors, n = 3
  - Lots pre-characterized for induction
- 3 day treatment at 7 concentrations (don't skimp!)
- Media drug concentration measurement
- Quantitative mRNA measurement by RT-PCR
  - CYP1A2, CYP2B6, CYP3A4)
- (Activity measurements, where applicable)
- Fold-change over vehicle control endpoint
- $EC_{50}/E_{max}$ determination, as applicable (more later)

# Supporting / preliminary assays

- 2 supporting experiments dictate test concentrations
  - Solubility testing for a high-concentration assay
    - Stock solvent solubility
    - Test system solubility (e.g., a variant of Modified Chee's Medium)
  - Cytotoxicity
    - Morphology observations
    - Lactate dehydrogenase release (LDH) assessment
- 1 more part is concurrent with the main assay
  - Spent media analysis / drug concentration analysis
    - Measuring [drug] over incubation time course on last day
    - What is it for?

# Cultured hepatocytes

- Cryopreserved vs. fresh
  - Scheduling
  - Characterization
  - Continuity while the lot lasts



- Options for characterization
  - What is standard characterization?
  - Do we need to use relative induction score (RIS) characterized lots?
    - It depends. More later…

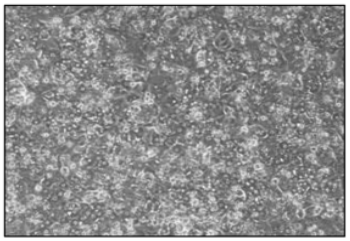
**H1500.H15A+ Lot No. HC3-45**

Cryopreserved Human Hepatocytes
Human, Female, Individual

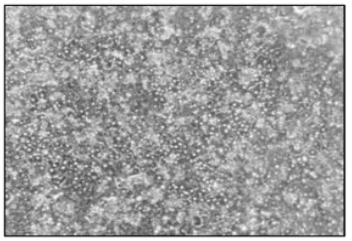
| | |
|---|---|
| Assured Minimum Yield: | 2.0 x $10^6$ cells per vial |
| Viability: | 83% |

Yield and viability are based on experiments performed at XenoTech using XenoTech's thawing protocol and OptiThaw Hepatocyte Kit.

Recommended Seeding Density: 1.4 million cells/mL



Photomicrograph (100x) of HC3-45 Day 2 of culture



Photomicrograph (100x) of HC3-45 incubation day

**Induction Data**

| Enzyme | Inducer | mRNA Fold Induction | Marker Substrate Reaction | Enzymatic Fold Induction |
|---|---|---|---|---|
| CYP1A2 | Omeprazole (50 µM) | 59.9 | Phenacetin O-dealkylation | 17.6 |
| CYP2B6 | Phenobarbital (750 µM) | 7.3 | Bupropion hydroxylation | 5.8 |
| CYP3A4 | Rifampin (20 µM) | 34.6 | Midazolam 1'-hydroxylation | 11.6 |

# Why not immortalized cell lines?

- Acceptable per FDA 2017
- Complementary per EMA 2013

- HepaRG
  - Low CAR expression
- Fa2N4
  - Lack functional CAR
  - Low basal CYP enzyme expression
- HepG2
  - High AhR expression
  - Low basal enzyme expression
  - Little/no response to known CYP3A4 inducers

"No one cell line affords an exact reproduction of a hepatocyte and most companies felt that these cell lines are not fully understood or characterized…we do not consider immortalized hepatocyte cell lines are an inadequate replacement for primary human hepatocytes"

Drug Metab Dispos. 2009 Jul;37(7):1339-54

# Target enzymes / prototypical inducers

- What are agency recommendations?

| Enzyme | Receptor | FDA | EMA |
|---|---|---|---|
| CYP1A2 | AhR | Omeprazole<br>Lansoprazole | Omeprazole |
| CYP2B6 | CAR | Phenobarbital | CITCO |
| CYP3A4<br>CYP2C8<br>CYP2C9<br>CYP2C19 | PXR | Rifampin | Rifampin |

- Why select phenobarbital for CYP2B6?
  - CITCO: selective for CAR, fold-change response is variable
  - Phenobarbital: consistent response, accounts for CAR/PXR crosstalk

FDA 2017 Guidance for Industry: In vitro metabolism- and transporter-mediated drug-drug interaction studies.

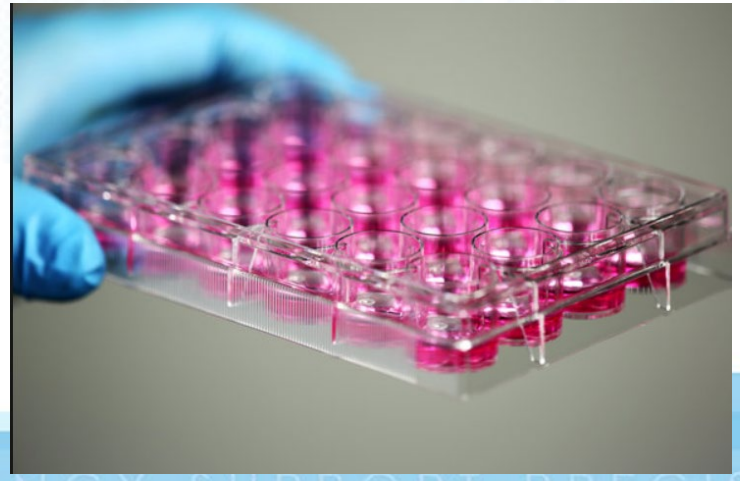
EMA 2013 Guideline on the investigation of drug interactions

Hariparsad *Drug Metab. Dispos.* 2017, 45: 1049-1059.

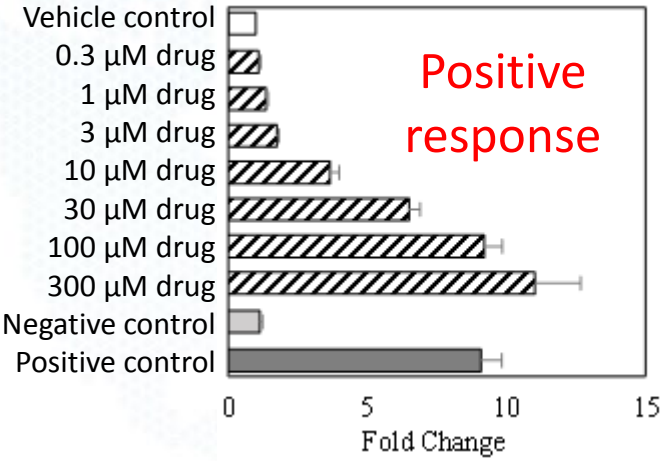
# Incubation

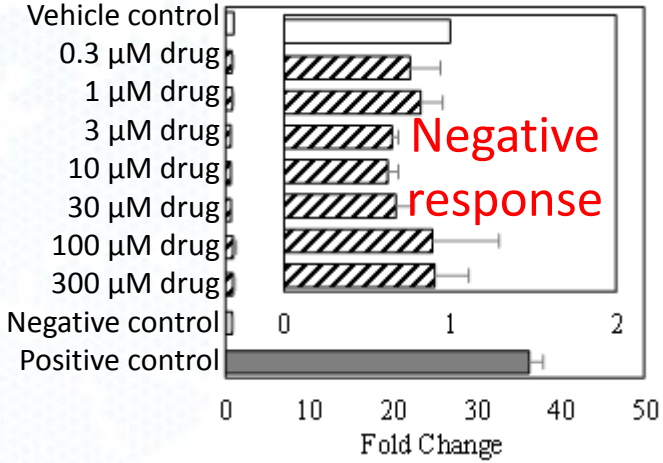
- Sandwich-cultured hepatocytes
- 1 day adaptation
- 3 days of treatment at multiple concentrations
- Sample collection out to 24-h post last treatment
- Collect spent media for analysis
- (Perform enzyme activity incubations)
- Collect cells for mRNA harvest
- Prepare samples for analysis
- Analyze samples
  - qRT-PCR
  - LC-MS/MS

Vehicle control
0.3 µM drug
1 µM drug
3 µM drug
10 µM drug
30 µM drug
100 µM drug
300 µM drug
Negative control
Positive control



# First-pass data: fold-change over vehicle control



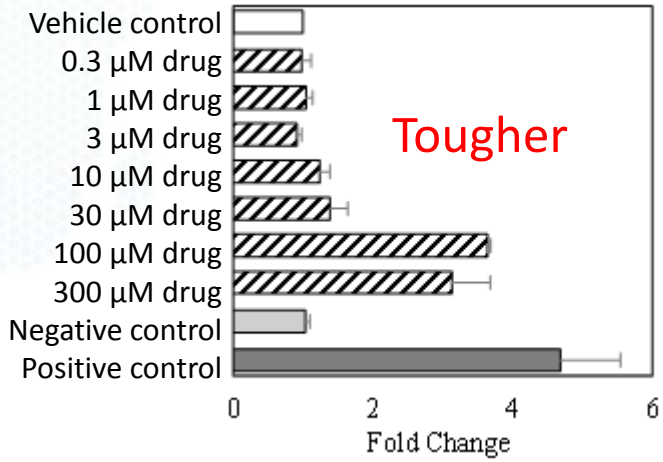




<u>POSITIVE</u>

≥ 2-fold change over the vehicle control

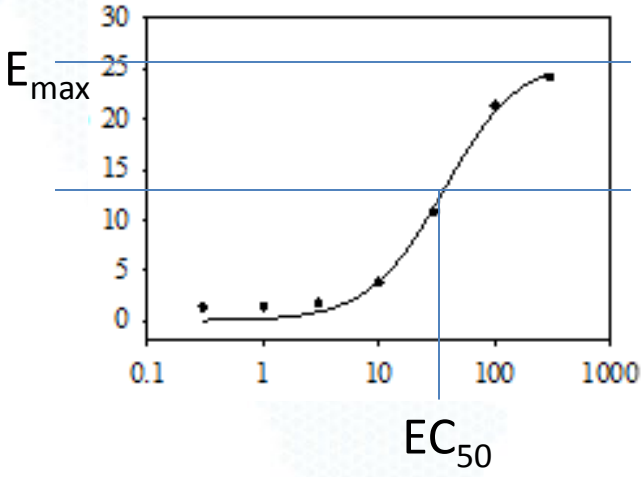
AND

≥ 20% of the positive control response



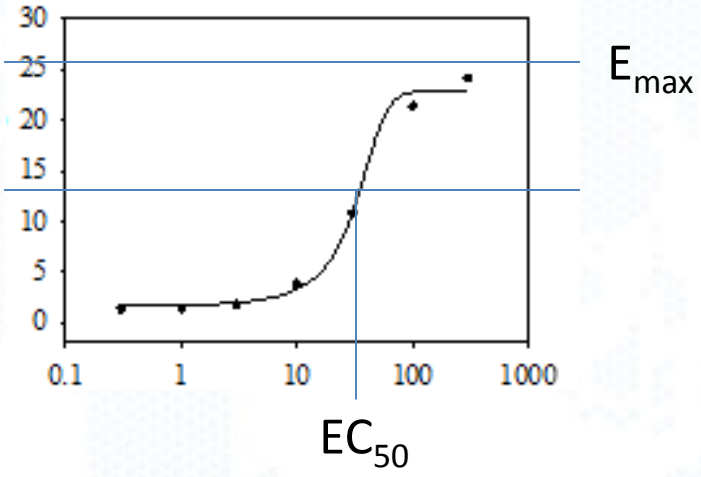
# Induction constants

- $EC_{50}$ – concentration associated with half-maximal induction
- $E_{max}$ – maximal fold induction in vitro




Sigmoid 3-parameter

$$f = \frac{a}{1 + e^{\left(\frac{-(x - x_0)}{b}\right)}}$$




Hill 3-parameter

$$f = \frac{a \times x^b}{\left(x_0^b + x^b\right)}$$

f = fold
a = $E_{max}$
$x_0$ = $EC_{50}$
x = drug concentration
b = slope (Hill coefficient)

# What does the data mean?

- What is a positive?
  - ≥ 2-fold change over vehicle control and 20% of the positive control response in ANY ONE DONOR
  - FDA and EMA agree now!
- Why is one donor response a positive?
  - Interindividual variability
  - Conservative approach to safety
- What do I do about it?
  - If CYP3A4, then CYP2C evaluation warranted
  - Mathematical risk assessment is a good next step
    - More later…
  - Fourth culture? Explored in IQ IWG 2018 paper

Kenny et al. *Drug Metab. Dispos.* 2018, 47(5): 1285-1303.

# CYP2C enzymes

- If CYP3A4 induction is observed, need to follow up with
  - CYP2C8
  - CYP2C9
  - CYP2C19
- All four enzymes can be induced through the PXR pathway
- mRNA or activity measurements?
  - mRNA fold-change highly variable for all CYP2Cs
  - mRNA rifampin fold-change often < 2
  - Activity is a more reliable endpoint; fold-change may still be < 2
  - Multiple possible reasons are given in IQ-IWG 2017 paper
- What about using a 'good' 2C lot?
  - Only use lots with evidence of 2C induction, but still not predictable

Hariparsad *Drug Metab. Dispos.* 2017, 45: 1049-1059.

# Data analysis for positive induction responses

- Mathematical tools for risk assessment
  - Correlation methods
  - Basic modeling
  - Static mechanistic modeling
  - Dynamic mechanistic modeling (Physiologically based pharmacokinetics - PBPK)

- Generally, need dose and $C_{max}$ data or projections
- There are options for situations before $C_{max}$ established

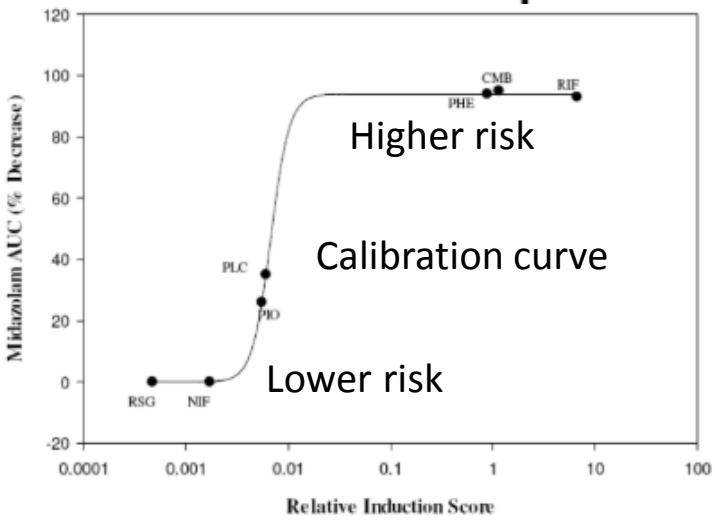
# Correlation methods

**Figure 3: Two Correlation Methods to Assess the Potential of an Investigational Drug to Induce Metabolizing Enzymes (Fahmi and Ripp, 2010)**

**Correlation Method 1**: Calculate a relative induction score (RIS) using $(E_{max} \times I_{max,u}) / (EC_{50} + I_{max,u})$
OR
**Correlation Method 2**: Calculate $I_{max,u} / EC_{50}$ values

Determine the magnitude of a clinical induction effect (e.g., AUC ratio of index substrate in the presence and absence of inducers) according to a calibration curve of RIS scores or $I_{max,u}/EC_{50}$ for a set of known inducers of the same enzyme.

$E_{max}$ is the maximum induction effect determined in vitro.
$EC_{50}$ is the concentration causing half-maximal effect determined in vitro.
$I_{max,u}$ is the maximal unbound plasma concentration of the interacting drug




- EMA allows the use of AUC/$F_2$ for correlation
- $F_2$ – the [inducer] leading to 2-fold increase of the baseline levels of enzyme
- Useful if $E_{max}/EC_{50}$ undefined

Fahmi and Ripp *Drug Metab. Dispos.* 2010, 6(11): 1399-1416.

# Basic modeling

**Figure 4: An Equation to Calculate the Predicted Ratio of the Victim Drug's AUC in the Presence and Absence of an Inducer for Basic Models of Induction**

$$R_3 = 1 / [1 + (d \times E_{max} \times 10 \times I_{max,u}) / (EC_{50} + (10 \times I_{max,u}))]$$

**$R_3$** is the predicted ratio of the victim drug's AUC in the presence and absence of an inducer for basic models of enzyme induction.
**d** is the scaling factor and is assumed to be 1 unless supported by prior experience with the system used.
**$E_{max}$** is the maximum induction effect determined in vitro.
**$I_{max,u}$** is the maximal unbound plasma concentration of the interacting drug.*
**$EC_{50}$** is the concentration causing half-maximal effect determined in vitro.

*Considering uncertainties in the protein binding measurements, the unbound fraction should be set to 1% if experimentally determined to be <1%.

If $R_3 \leq 0.8$, further investigation is warranted

Einolf et al. *Clin. Pharmacol. Ther.* 2014, 95(2): 179-188.

# Static mechanistic modeling

$$AUCR = \left( \frac{1}{[A_g \times B_g \times C_g] \times (1 - F_g) + F_g} \right) \times \left( \frac{1}{[A_h \times B_h \times C_h] \times f_m + (1 - f_m)} \right)$$

No net effect model now: Why??

**A** is the effect of reversible inhibitions.
**B** is the effect of TDI.
**C** is the effect of induction.
$\mathbf{F_g}$ is the fraction available after intestinal metabolism.
$\mathbf{f_m}$ is the fraction of systemic clearance of the substrate mediated by the CYP enzyme that is subject to inhibition/induction. Each value can be estimated with the following equations:

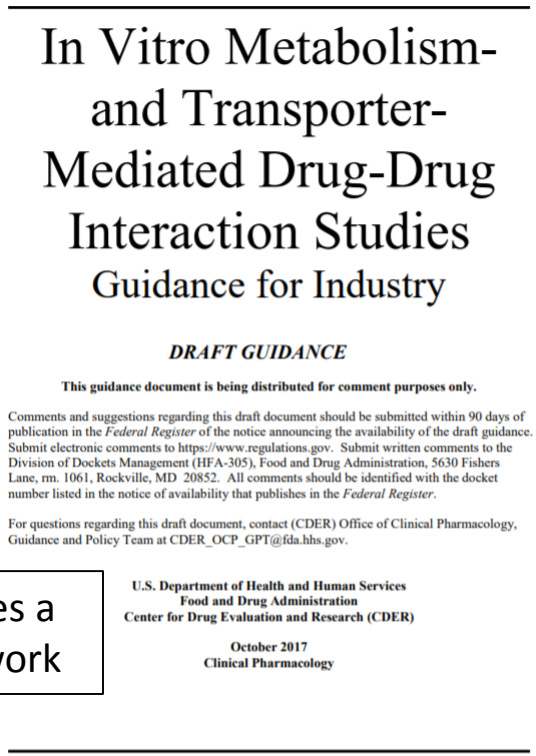
| | Gut | Liver |
|---|---|---|
| Reversible inhibition | $A_g = \frac{1}{1 + \frac{[I]_g}{K_i}}$ | $A_h = \frac{1}{1 + \frac{[I]_h}{K_i}}$ |
| Time-dependent inhibition | $B_g = \frac{k_{deg,g}}{k_{deg,g} + \frac{[I]_g \times k_{inact}}{[I]_g + K_I}}$ | $B_h = \frac{k_{deg,h}}{k_{deg,h} + \frac{[I]_h \times k_{inact}}{[I]_h + K_I}}$ |
| Induction | $C_g = 1 + \frac{d \bullet E_{max} \bullet [I]_g}{[I]_g + EC_{50}}$ | $C_h = 1 + \frac{d \bullet E_{max} \bullet [I]_h}{[I]_h + EC_{50}}$ |

If AUC ratio ≤ 0.8, further investigation is warranted

Fahmi et al. *Drug Metab. Dispos.* 2009, 37(8): 1658-1666.

# Dynamic mechanistic modeling: PBPK

- Prediction of the DDI potential as a substrate or a perpetrator



In Vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies
Guidance for Industry

***DRAFT GUIDANCE***

**This guidance document is being distributed for comment purposes only.**

Comments and suggestions regarding this draft document should be submitted within 90 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit electronic comments to https://www.regulations.gov. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register*.

For questions regarding this draft document, contact (CDER) Office of Clinical Pharmacology, Guidance and Policy Team at CDER_OCP_GPT@fda.hhs.gov.

**U.S. Department of Health and Human Services**
**Food and Drug Administration**
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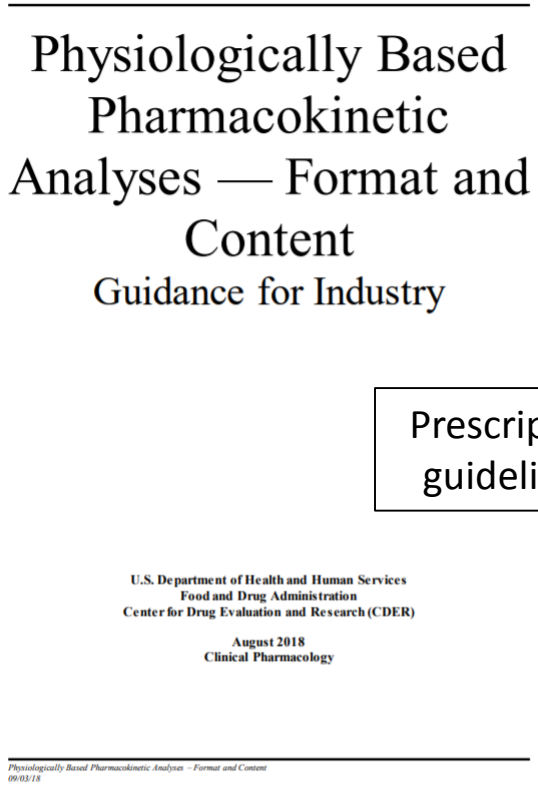
**October 2017**
**Clinical Pharmacology**

*10/24/17*

Provides a framework



Physiologically Based Pharmacokinetic Analyses — Format and Content
Guidance for Industry

**U.S. Department of Health and Human Services**
**Food and Drug Administration**
**Center for Drug Evaluation and Research (CDER)**

**August 2018**
**Clinical Pharmacology**

*Physiologically Based Pharmacokinetic Analyses – Format and Content*
*09/03/18*

Prescriptive guidelines

# Unanswered questions

- When should I place the study?
- Do I need a negative control?
- Can I use my validated method for the concentration measurements?

- More complex questions….

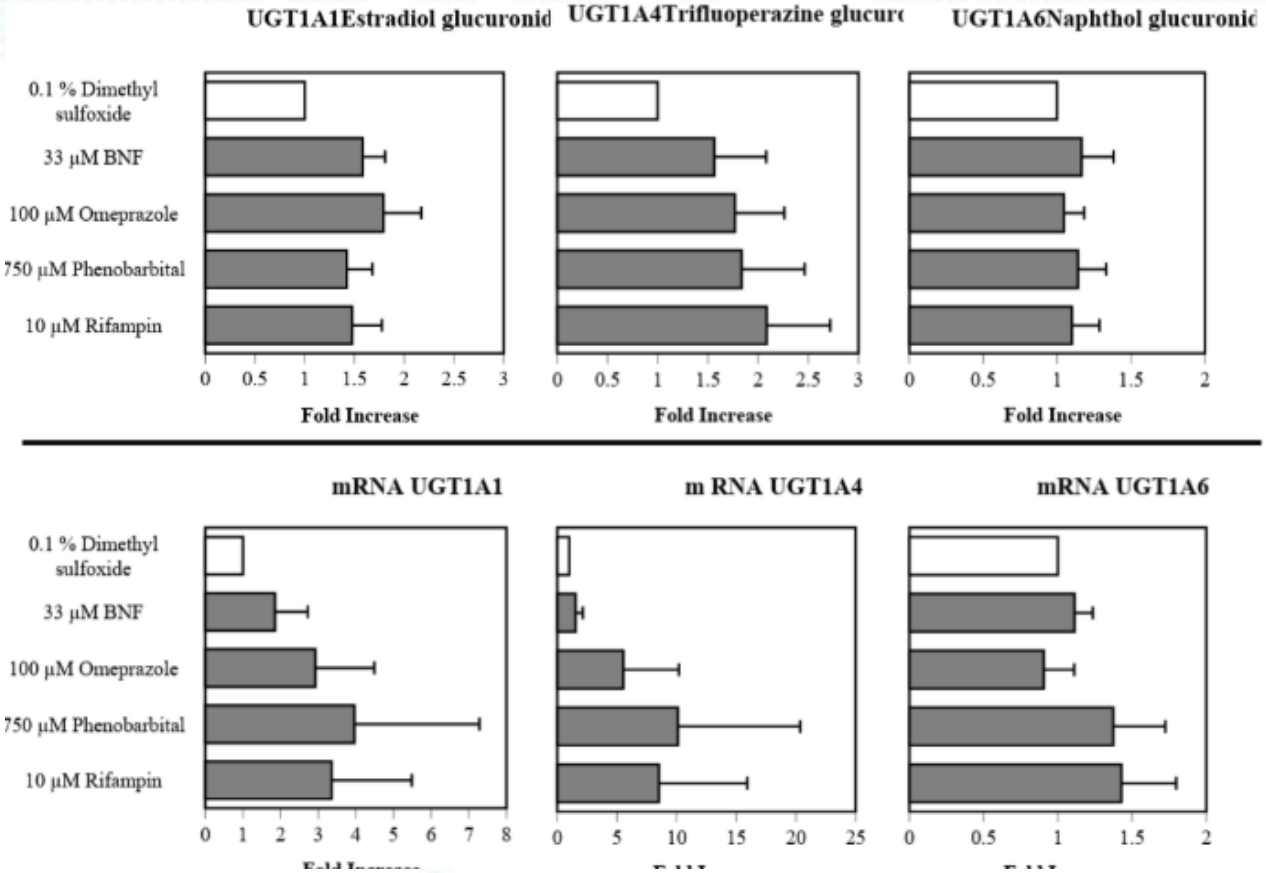
# What if there is down-regulation?

- Down-regulation does not equal clinical effects
- EMA cutoff: 50% decrease and concentration-dependence
- Evaluate relationship with
  - Mechanism of action
  - Cytotoxicity
  - Enzyme inhibition (activity measurements)
- Mechanistic studies may be needed to evaluate cause
  - May be a big undertaking
  - Scarcity of useful research in this area

IQ-IWG 2017:
Hariparsad et al. *Drug Metab. Dispos.* 2017, 45: 1049-1059.

# Induction: UGT enzymes and transporters

- UGT induction usually less problematic in vivo than CYP, but…




FDA, 2017:
"In vitro methods to evaluate induction of P-gp and other transporters are not well-established. Therefore the FDA does not currently recommend in vitro evaluation of transporter induction"

FDA 2017 Guidance for Industry: In vitro metabolism- and transporter-mediated drug-drug interaction studies.

# Conclusions

- Study designs should meet
  - Scientific needs
  - Regulatory needs
- A positive result (even from one donor)
  - Means some form of follow up is needed
  - Does not necessarily lead straight to the clinic
- Science is still science and some aspects are not perfect
- Make decisions carefully based on the program

- Client questions are rigorously discussed internally!

# Acknowledgments


- Rebecca Campbell
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- Dr. Brian Ogilvie

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