PROVEN GLOBAL CONTRACT RESEARCH EXPERTISE
FROM DISCOVERY THROUGH CLINICAL SUPPORT

In Vitro Inhibition Studies:

Elements of Design and Important Considerations in Data Analysis

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Frequently Asked Questions

- Why is measuring CYP inhibition important?
- Where do I start?
- How much material is needed?
- Results: What do my data mean?

Why is measuring CYP inhibition important?

- Cytochromes P450 (CYPs): membrane-bound, heme-containing enzymes

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- Perpetrator or precipitant: the drug causing the interaction – the drug is an inhibitor of drug-metabolizing enzymes.

- Victim or object: the affected drug – the drug is a substrate of one or more affected enzymes

Why is measuring CYP inhibition important?

Victims	Perpetrators
Clearance determined by a single route of elimination	Factors that alter the clearance of a victim drug
Victim drugs – Not approved Debrisoquine, perhexilline	⇐ **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Victim drugs – Withdrawn Terfenadine, cisapride, astemizole and cerivastatin	⇐ **Inhibitory drugs** Erythromycin, ketoconazole mibefradil (withdrawn)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) may deny approval (AIDS patients)
Victims – PK and toxicity Digoxin, bile salts, statins, fexofenadine	⇐ **Transporter interactions** Quinidine, bosentan, cyclosporin A, grapefruit juice

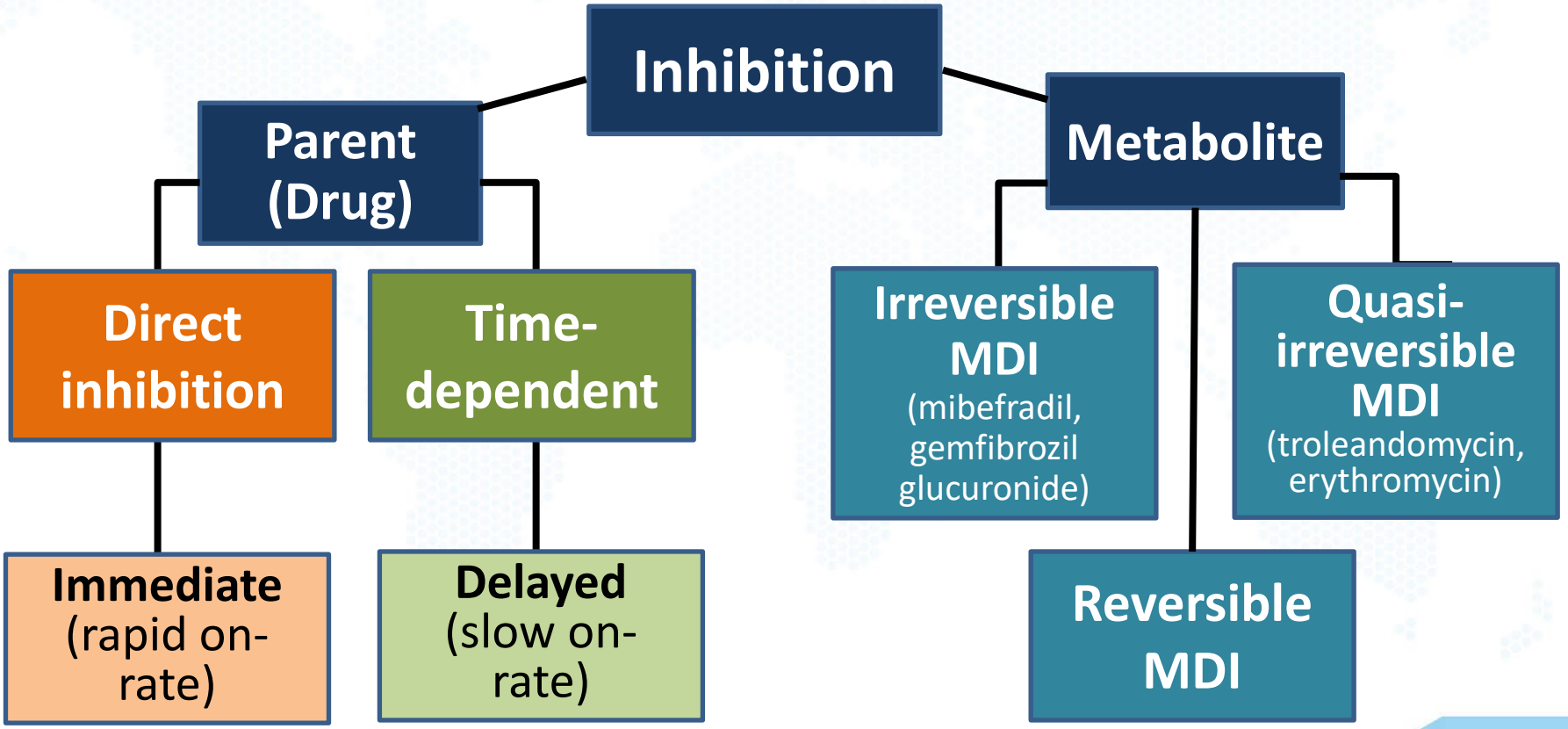
The Major Human Xenobiotic-Metabolizing CYPs

CYP1A2
CYP2B6
CYP2C8
CYP2C9
CYP2C19
CYP2D6
CYP3A4
CYP3A5

- A subset of the 55 functional human CYPs
- Often catalyze the rate-limiting step in the elimination of numerous xenobiotics
- Highly expressed in the liver (but present in many tissues)
- CYP3A4 is also highly expressed in the small intestine
- Inhibition can lead to increased exposure to xenobiotics – or decreased activation of toxicant or formation of active metabolites
- A single xenobiotic can inhibit multiple CYPs, potentially leading to large magnitude increases in exposure

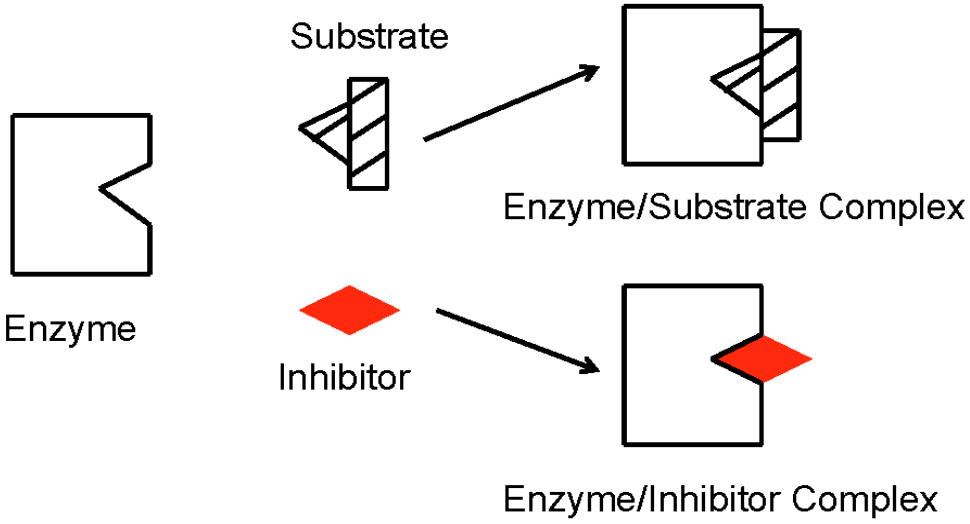
CYP2D6 and **3A4** metabolize ~40% of drugs and 20% of natural products and endobiotics

Types of enzyme inhibition



Direct inhibition

- The inhibitor and substrate bind to the same site on the enzyme.
- Also referred to as 'reversible' inhibition
  - Reversible means that the inhibitor is free to come and go from the active site.



Example:
Competitive inhibition

Time-dependent inhibition (TDI)

- Caused by test article/drug
- Does not require NADPH
- Delayed onset of inhibition
  - Often seen with acidic drugs, oligonucleotides
- Metabolism by non-NADPH-dependent enzymes (e.g., MAO, carboxylesterases)
- Non-enzymatic degradation

Metabolism-dependent inhibition (MDI)

- Requires NADPH
- Due to metabolism by CYP or NADPH-dependent enzymes
- Delayed because metabolites have to be generated first in order to cause inhibition
- Irreversible MDI persists until new enzyme is synthesized

Where do I start?

IC_{50} determination

- SXT's CYP design measures in one assay:
  1. Direct (Zero-min preincubation)
  2. TDI (30-min preincubation *without* NADPH)
  3. MDI (30-min preincubation *with* NADPH)
- Quantitative assay (how much inhibition)
- IC_{50} = concentration of inhibitor causing 50% inhibition of enzyme activity
- Controls = no solvent control, solvent control and positive control inhibitors for direct and metabolism-dependent inhibition

IC_{50} determination

- 7 test article concentrations, [protein] ≤ 0.1 mg/mL
  - Protein concentration is low to minimize inhibitor depletion and membrane partitioning ("binding")
- Substrate incubation time is short relative to the preincubation time (i.e., 5 min incubation vs 30 min preincubation)
  - Ensures that we are only capturing direct inhibition for 0-min preincubation samples
- Performed at 1 substrate concentration (~ K_m or S_{50})
  - Needs to be run under initial rate conditions (less than 20% of substrate added is metabolized)

Substrates

Enzyme	Marker reaction
CYP1A2	Phenacetin O-deethylation, 7-Ethoxyresorufin-O-deethylation
CYP2B6	Efavirenz hydroxylation, Bupropion hydroxylation
CYP2C8	Paclitaxel 6α-hydroxylation, Amodiaquine N-deethylation
CYP2C9	S-Warfarin 7-hydroxylation, Diclofenac 4'-hydroxylation
CYP2C19	S-Mephenytoin 4'-hydroxylation
CYP2D6	Bufuralol 1'-hydroxylation, Dextromethorphan O-demethylation
CYP3A4/5*	Midazolam 1'-hydroxylation, Testosterone 6β-hydroxylation

* Recommend the use of 2 structurally unrelated CYP3A4/5 substrates for evaluation of in vitro CYP3A4/5 inhibition.

https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers#table1 Tables 1-1 and 1-2

How much test material is needed?

https://www.xenotech.com/webinar-videos/calculate-in-vitro-dmpk-ddi-study-test-article

Test article Properties

- **Molecular weight**—the larger the molecular weight, the more compound you need to prepare a given concentration of solution
- **Route of administration**– Regulatory guidance specifies different parameters that are dependent upon how the test article is administered
- **Dose (if oral)** – more compound ingested results in a higher concentration of exposure in the gut
- **C_{max}** – Maximum drug concentration in the plasma at steady state provides information on the anticipated systemic exposure
- **Plasma free fraction** – Compounds that are more abundantly protein bound have decreased bioavailability
- **LogP** – Partition coefficient

Calculations that define the highest incubation concentration to be evaluated

$0.1 \times I_{gut}$

I_{gut} = Apparent intestinal luminal concentration

Concentration equal to the maximum dose in 250 mL (a glass of water)

Applicable for enzymes that are highly expressed in the intestine compared to other tissues within the body

E.g., CYP3A4/5

50 x Unbound C_{max} at steady state

Cmax = Maximal drug concentration in the plasma

If fraction unbound is unknown a conservative approach is taken (assume 100% unbound)

Applicable for systemic concentrations or ubiquitously expressed enzymes

E.g., CYP1A2 (non-CYP3A4/5 enzymes)

Solvent Considerations

- Some organic solvents can inhibit or activate enzymes at high concentrations
- FDA guidance states that the sponsor should keep organic solvents at low concentrations (<1% v/v and preferably <0.5% v/v) in final incubation
- For test articles used in CYP inhibition studies, XenoTech limits all organic solvent percentages to 0.2% (DMSO) to 1% v/v (methanol / acetonitrile)
    - test article solubility in the test system permitting

Solubility considerations

"My drug is not very soluble in aqueous solution."

- Can you test pharmacologically relevant concentrations?

  FDA guidance does allow for evaluation at **the limit of aqueous solubility**

- Can you include BSA to improve the solubility?

  Compounds that are not soluble in aqueous solutions may be more likely to bind to proteins

  BSA may improve solubility but it comes at the price of reduction in free concentration

Results: what do my data mean?

CYP inhibition decision tree

Evaluation of direct, time- and metabolism-dependent inhibition (IC_{50} determination)
Multiple [inhibitor] x single [substrate]
No preincubation and 30-min preincubation ± NADPH

Direct inhibition
(Low IC_{50} value)

Predict K_i value
Determine the affinity of an inhibitor for an enzyme

Time-dependent
(Decrease in IC_{50} after 30-min preincubation ***without*** NADPH)

Evaluate non-enzymatic degradation and/or metabolism by non-NADPH-dependent enzymes

Metabolism-dependent
(Decrease in IC_{50} after 30-min preincubation ***with*** NADPH)

XenoTech Reversibility Assay
(Ultracentrifugation method to evaluate mechanism of MDI)

Reversible

No further studies

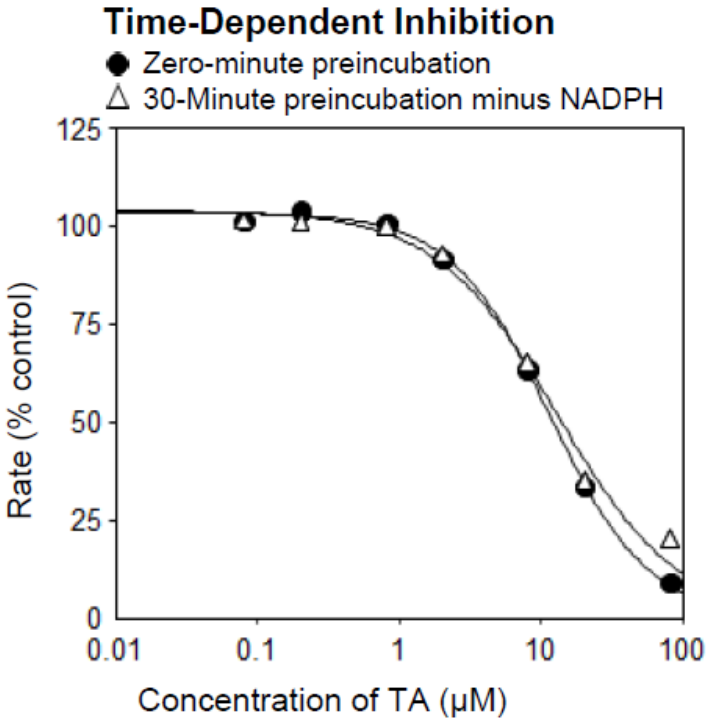
Quasi- or Irreversible

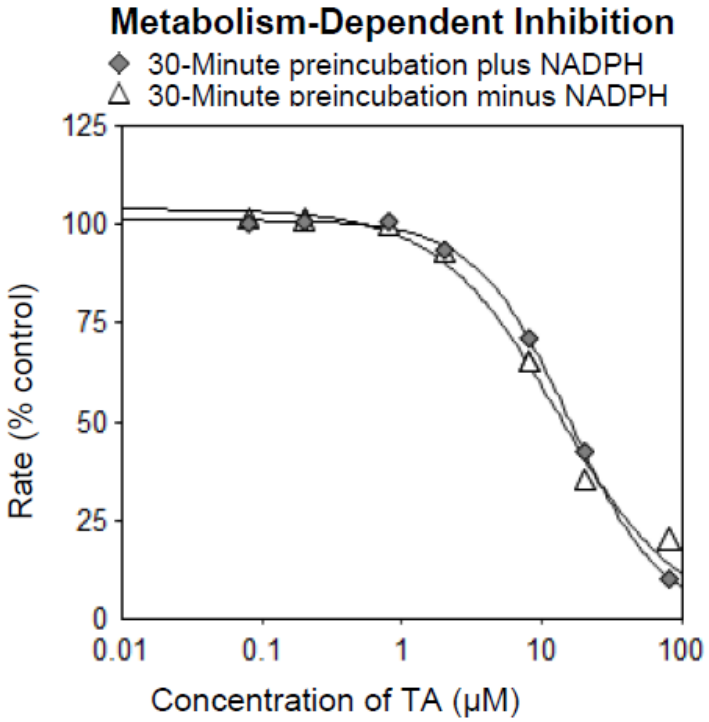
Measure k_{inact} and K_I
Multiple [I] x multiple preincubation times

Note that ***Ki*** (lower case 'i') for direct inhibition is not the same as ***KI*** (upper case 'I') for MDI

IC_{50} determination – CYP inhibition

CYP2D6 (Dextromethorphan O-demethylation)





Parameter: 4PL	Zero-minute preincubation (value ± standard error)	30-Minute preincubation minus NADPH (value ± standard error)	30-Minute preincubation plus NADPH (value ± standard error)
IC_{50} (µM)	11 ± 0	13 ± 2	16 ± 0
Slope	1.2 ± 0.1	1.0 ± 0.1	1.3 ± 0.0

Importance of K_i value

Figure 1: Equations to Calculate the R value for Basic Models of Reversible Inhibition (Vieira, Kirby, et al. 2014)

$$R_1 = 1 + (I_{max,u} / K_{i,u})$$

$$R_{1,gut} = 1 + (I_{gut} / K_{i,u})$$

$I_{max,u}$ is the maximal unbound plasma concentration of the interacting drug at steady state.*
I_{gut} is the intestinal luminal concentration of the interacting drug calculated as the dose/250 mL.
$K_{i,u}$ is the unbound inhibition constant determined in vitro.

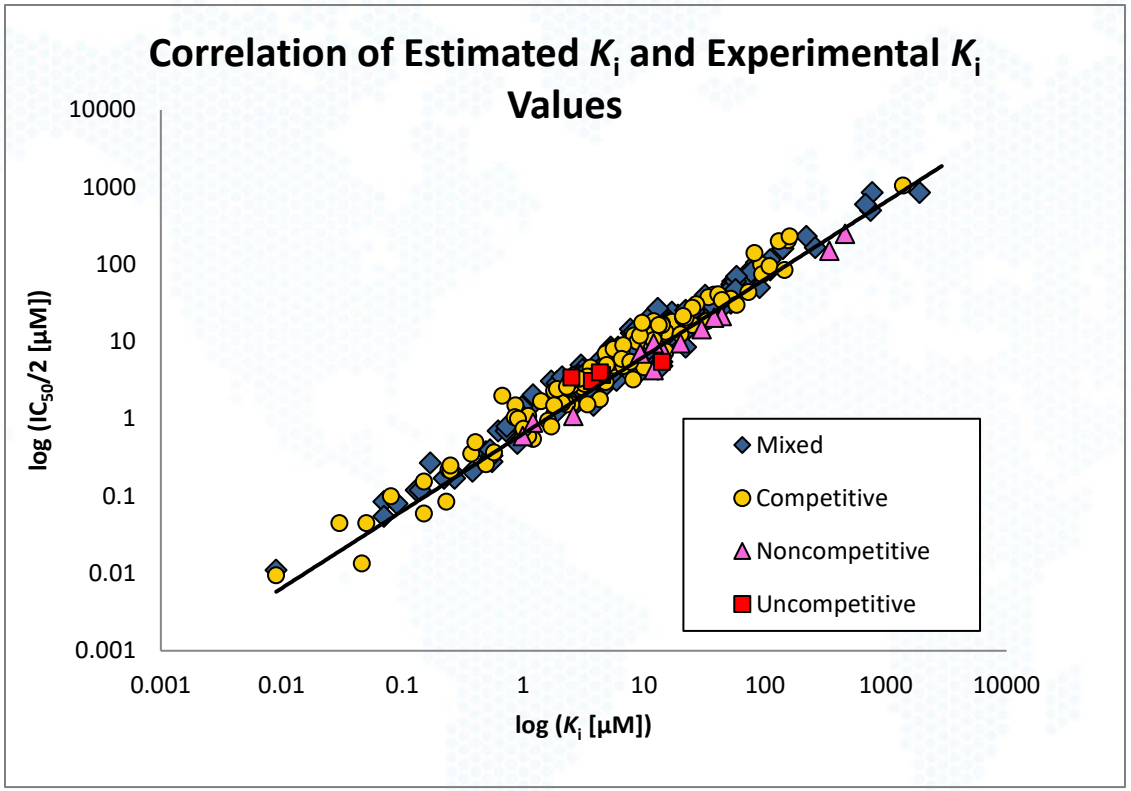
Note: I and K_i need to be expressed in the same unit (e.g., in a molar concentration unit).

*Considering uncertainties in the protein binding measurements, the unbound fraction in plasma should be set to 1% (fraction unbound in the plasma ($f_{u,p}$) = 0.01) if experimentally determined to be < 1%.

- If $R_1 \geq 1.02$ or $R_{1,gut} \geq 11$, 'the sponsor should further investigate the DDI potential by either using mechanistic models or conducting a clinical DDI study. . .'

[FDA] Food and Drug Administration (2020) Guidance for industry: In vitro drug interaction studies - Cytochrome P450 enzyme- and transporter-mediated drug interactions, U.S. Department of Health and Human Services, Rockville, MD. 43 p.

Predicted K_i value



$K_i = IC_{50}/2$ when [S] = Km

This is now cited in the final 2020 US FDA guidance

Based on the **Cheng-Prusoff equation** for competitive inhibition: Cheng and Prusoff (1973). Biochem Pharmacol 22:3099.

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

Haupt LJ, Kazmi F, Ogilvie B, Buckley DB, Smith BD, Leatherman S, Paris B, Parkinson O and Parkinson A (2015) The reliability of estimating Ki values for direct, reversible inhibition of cytochrome P450 enzymes from corresponding IC50 values: A retrospective analysis of 343 experiments. *Drug Metab Dispos* 43:1744-1750.

CYP inhibition decision tree

Evaluation of direct, time- and metabolism-dependent inhibition (IC_{50} determination)
Multiple [inhibitor] x single [substrate]
No preincubation and 30-min preincubation ± NADPH

Metabolism-dependent
(Decrease in IC_{50} after 30-min preincubation ***with*** NADPH)

XenoTech Reversibility Assay
(Ultracentrifugation method to evaluate mechanism of MDI)

Quasi- or Irreversible

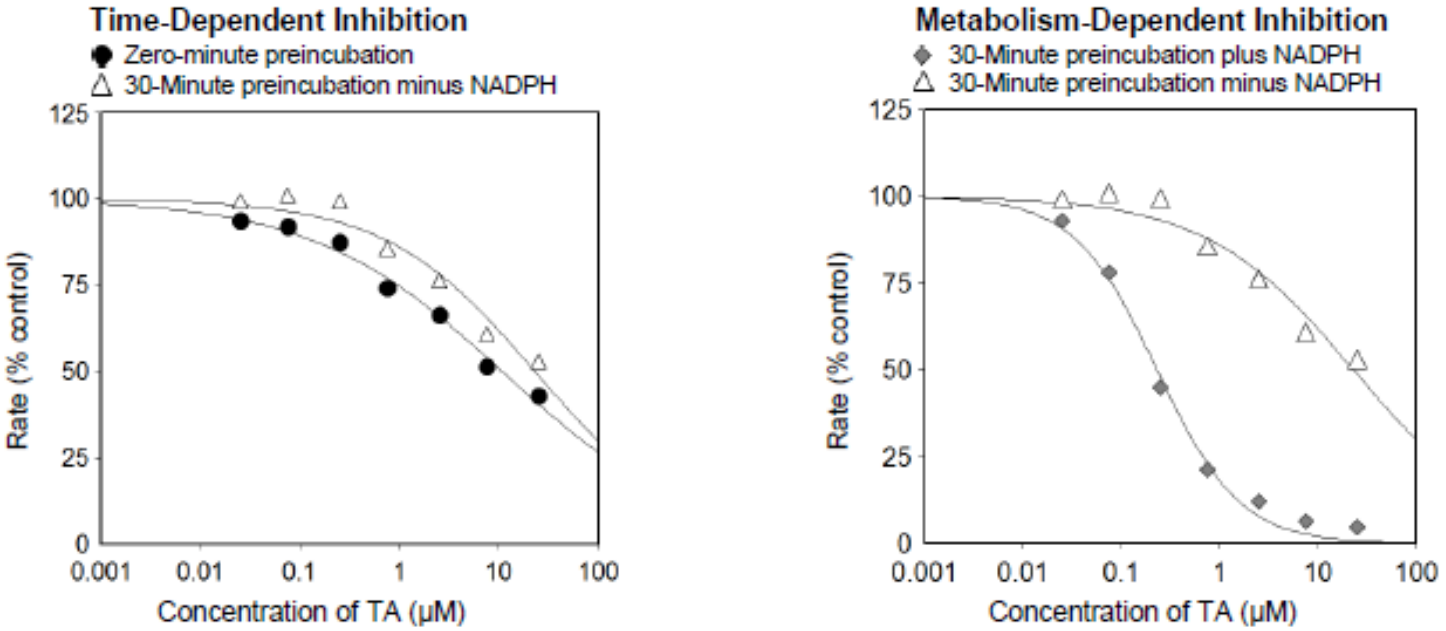
Measure k_{inact} and K_I
Multiple [I] x multiple preincubation times

Reversible

No further studies

IC_{50} determination – CYP inhibition

CYP3A4/5 (Midazolam 1´-hydroxylation)



Parameter: 4PL	Zero-minute preincubation (value ± standard error)	30-Minute preincubation minus NADPH (value ± standard error)	30-Minute preincubation plus NADPH (value ± standard error)
IC_{50} (μM)	11 ± 1	23 ± 6	0.23 ± 0.02
Slope	0.45 ± 0.03	0.58 ± 0.09	1.0 ± 0.1

Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KH, Lu C, Nomeir AA, Seibert E, Skordos KW, Tonn GR, Van Horn R, Wang RW, Wong YN, Yang TJ and Obach RS (2009) The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: A perspective of the Pharmaceutical Research and Manufacturers of America (PhRMA). *Drug Metab Dispos* 37(7):1355-1370.

Types of MDI

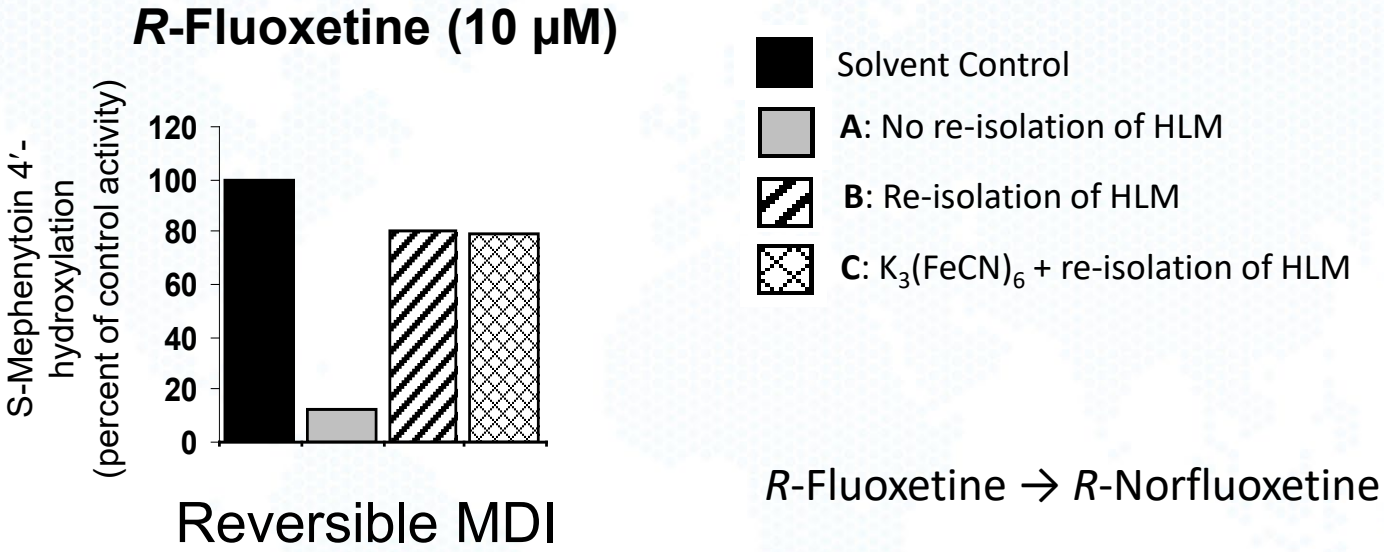
- **Reversible MDI**: metabolite is simply a more potent direct acting (or *reversible*) inhibitor
  - *R*-fluoxetine (CYP2C19)
- **Irreversible MDI**: metabolite covalently binds to the enzyme or active site
  - Mibefradil (CYP3A4/5), furafylline (CYP1A2), tienilic acid (CYP2C9), gemfibrozil glucuronide (CYP2C8), ticlopidine (CYP2C19)
- **Quasi-irreversible MDI**: metabolite coordinates very tightly to the heme iron of cytochrome P450
  - Troleandomycin (CYP3A4/5), *S*-fluoxetine (CYP2C19), erythromycin (CYP3A4/5)

XenoTech Reversibility Assay (XTRA)

- Qualitative assay (what type of inhibition)
- One concentration of test article is preincubated with HLM [0.1 mg/mL] anywhere from 30 to 120 min
- Unmetabolized test article and reversible inhibitory metabolites are removed from human liver microsomes by ultracentrifugation
- 3 typical groups of samples are prepared prior to measuring residual enzyme activity:
  - Group A: Preincubated only (replicates IC_{50})
  - Group B: Preincubated and microsomes are re-isolated
  - Group C: Preincubated; microsomes are treated with 2 mM $K_3Fe(CN)_6$ (to dissociate quasi-irreversible metabolite-inhibitor complexes) prior to centrifugation
- Marker substrate = 10 x K_m, solubility permitting

XenoTech Reversibility Assay (XTRA)

- Is MDI reversed by ultracentrifugation?

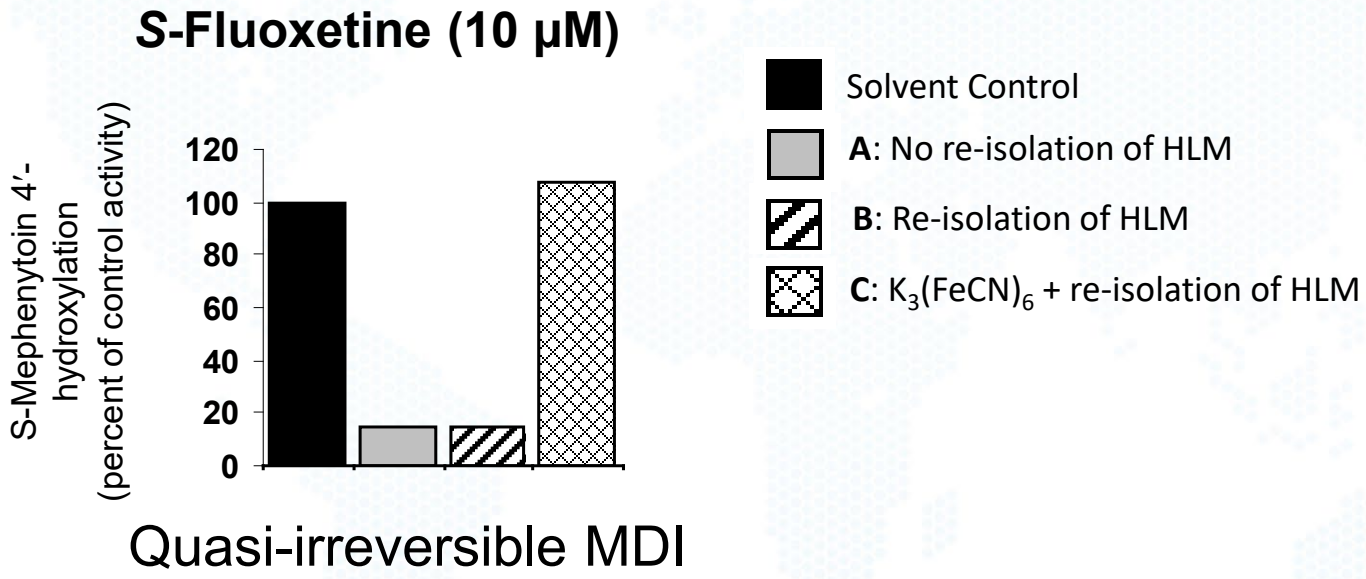


R-Fluoxetine → *R*-Norfluoxetine

- Yes: Metabolite is more potent *reversible* (direct) inhibitor than parent (reversible MDI)

XenoTech Reversibility Assay (XTRA)

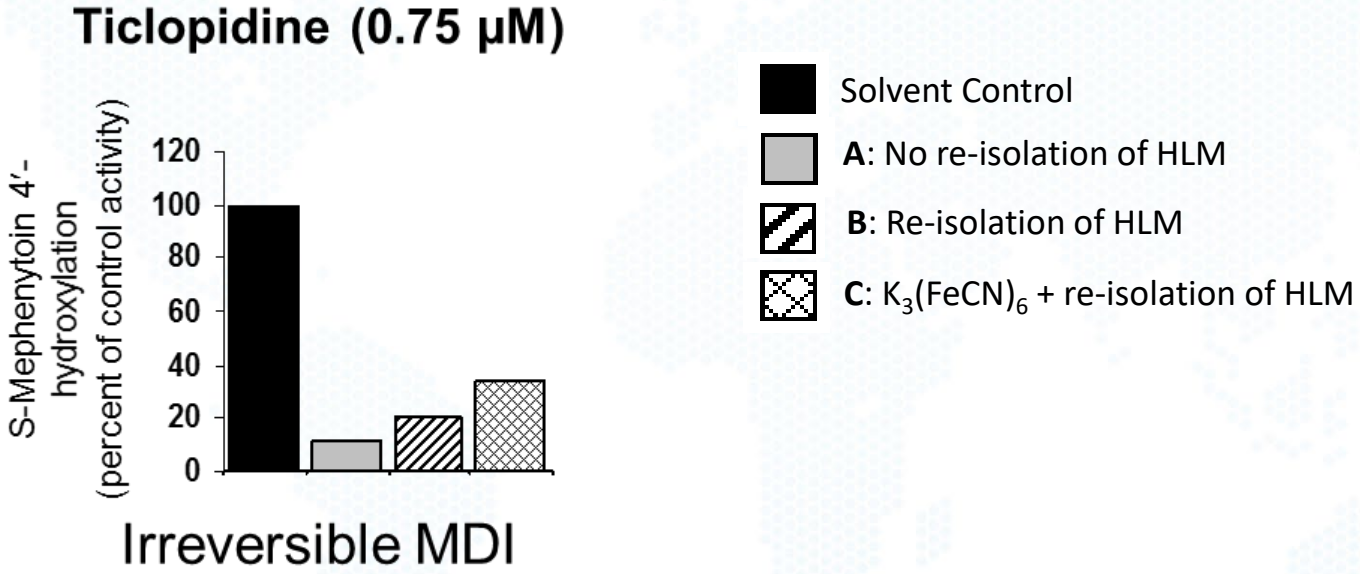
- Is MDI reversed by ultracentrifugation?
- Is MDI reversed by $K_3Fe(CN)_6$?



- MDI is likely due to inactivation that is reversed by $K_3Fe(CN)_6$ (quasi-irreversible)

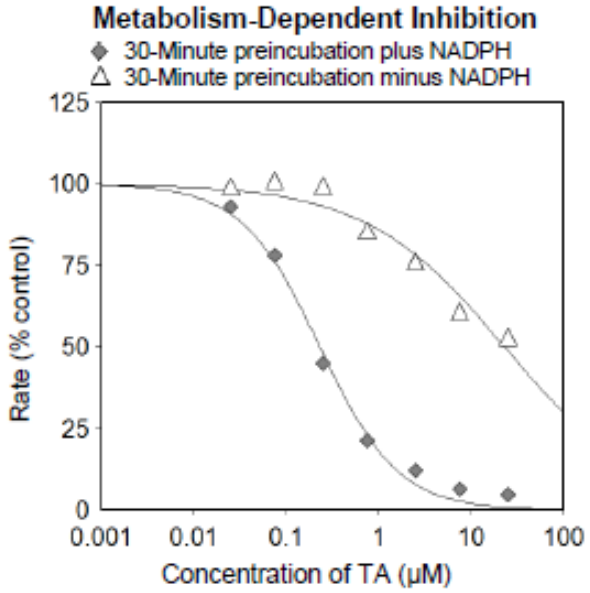
XenoTech Reversibility Assay (XTRA)

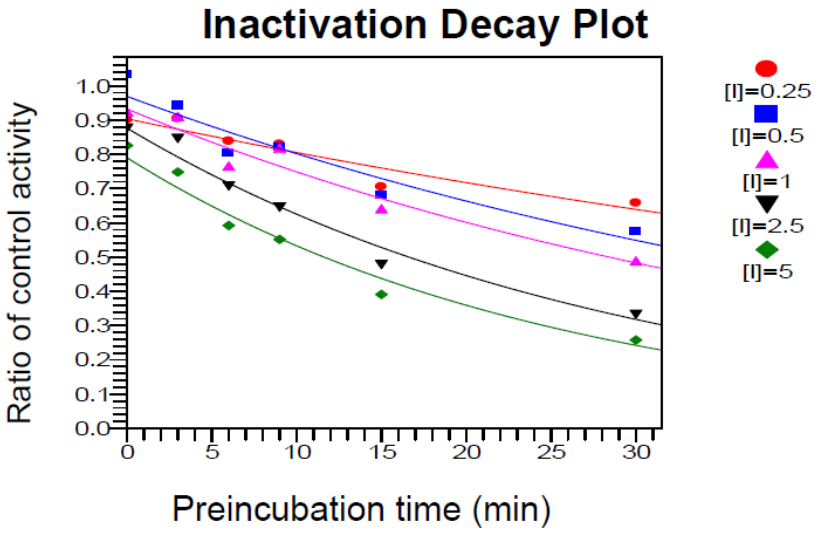
- Is MDI reversed by ultracentrifugation?
- Is MDI reversed by $K_3Fe(CN)_6$?



- MDI is likely due to inactivation NOT reversed by $K_3Fe(CN)_6$ (irreversible)

Determination of K_I and k_{inact}





IC_{50} is only a snapshot, usually after 30 min

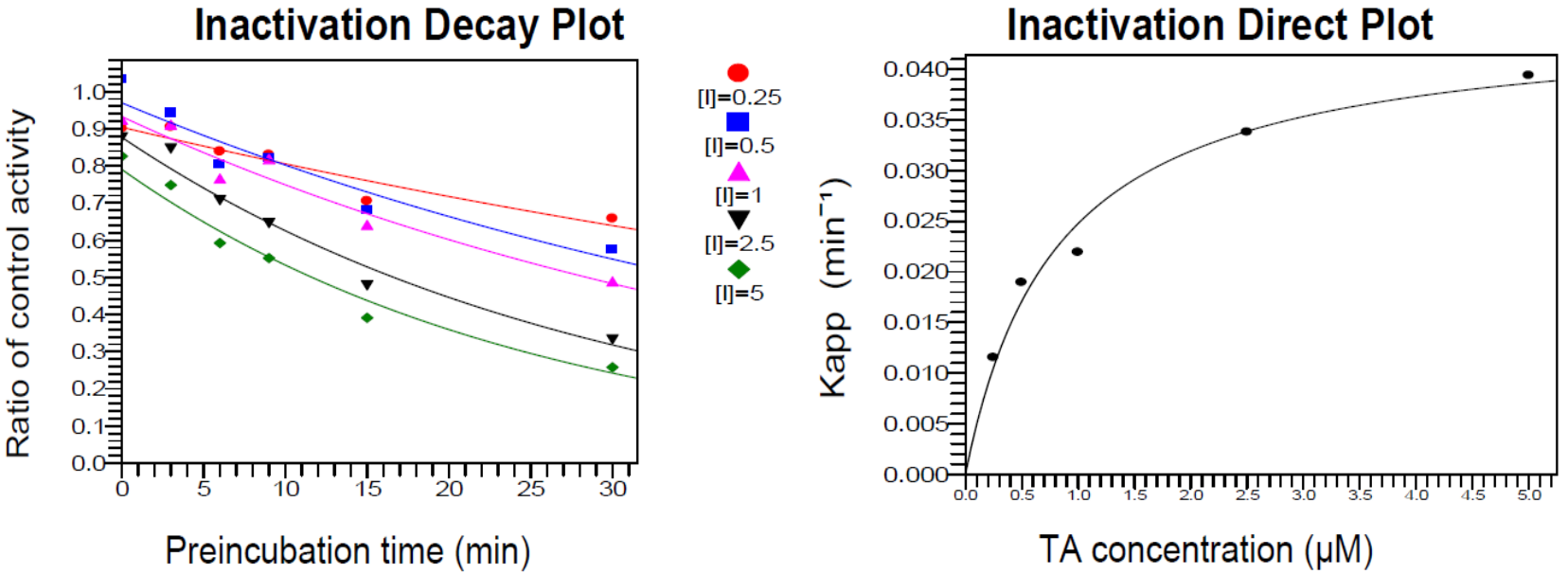
k_{inact} gives a broader picture of what is happening over the entire preincubation period

Determination of K_I and k_{inact}

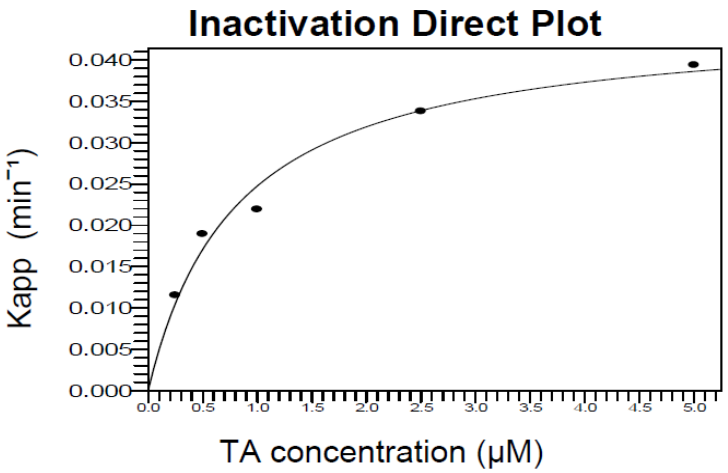
- Quantitative measurement of MDI
- 5 inhibitor concentrations + solvent control
- 5 preincubation time points + zero-min preincubation
- Must be performed at saturating marker substrate concentrations (e.g., 10 x K_m)
- Usually incorporates a 10- to 20-fold dilution

Determination of K_I and k_{inact}

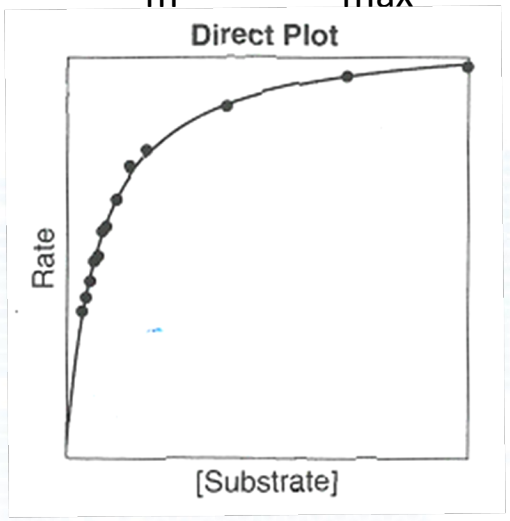
CYP3A4/5 (Midazolam 1´-hydroxylation)



Method: Two-step Nonlinear	Value ± standard error
K_I (µM)	0.81 ± 0.16
k_{inact} (min^{-1})	0.045 ± 0.003
k_{inact}/K_I (min^{-1} mM^{-1})	55



K_m and V_{max}



- k_{inact} for inactivation is analogous to Vmax for metabolism
- K_I is analogous to K_m for metabolism
- k_{inact}/K_I is the inactivation efficiency (similar to in vitro clearance, V_{max}/K_m)

Importance of K_I and k_{inact} values

For basic models of TDI, the sponsor should calculate R_2 as described in Figure 2.

Figure 2: Equations to Calculate the R value for Basic Models of TDI (Yang, Liao, et al. 2008; Grimm, Einolf, et al. 2009; Vieira, Kirby, et al. 2014)

$$R_2 = (k_{obs} + k_{deg}) / k_{deg}$$

$$\text{Where } k_{obs} = (k_{inact} \times 50 \times I_{max,u}) / (K_{I,u} + 50 \times I_{max,u})$$

k_{obs} is the observed (apparent first order) inactivation rate of the affected enzyme.
k_{deg} is the apparent first-order degradation rate constant of the affected enzyme.
$K_{I,u}$ is the unbound inhibitor concentration causing half-maximal inactivation.
k_{inact} is the maximal inactivation rate constant.
$I_{max,u}$ is the maximal unbound plasma concentration of the interacting drug at steady state.*

Note: I and K_I need to be expressed in the same unit (e.g., in a molar concentration unit).

*Considering uncertainties in the protein binding measurements, the unbound fraction in plasma should be set to 1% (fraction unbound in the plasma ($f_{u,p}$) = 0.01) if experimentally determined to be < 1%.

- 'If $R_2 \geq 1.25$, 'the sponsor should further investigate the DDI potential by either using mechanistic models or conducting a clinical DDI study. . .'

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Conclusion

- Negative data may obviate the need for clinical DDI studies
- Positive data can identify which DDIs should be investigated clinically and can assist in design of studies
- Positive data: potentially restricts co-medications during clinical trials
- Labeling implications: guidance on dosage adjustments or concentrations for co-medications
- Improves the safety profile of drugs and other registered xenobiotics

Our sponsors

Dr. Brian Ogilvie & Our scientific staff

Lois Haupt

Marketing department & ADME 101 presenters