

## Cytochrome P450 Inhibition

### Frequently Asked Questions

#### Why do I need to test to determine whether my drug candidate is a cytochrome P450 inhibitor?

Cytochrome P450 inhibition is a principle mechanism to pharmacokinetic drug-drug interactions. In vitro tests for cytochrome P450 inhibition have been shown to be reasonably predictive of the potential to cause these interactions. Regulatory agencies have issued guidance to industry regarding when and how to conduct these tests.

#### How is cytochrome P450 inhibition measured?

There are many variations of this measurement but they all use a source of active cytochrome P450 enzyme and a probe substrate which is metabolized by the cytochrome P450 to a specific metabolite. An inhibitor is added at varying concentrations and the effect of the different inhibitor concentrations on cytochrome P450 activity is determined by analyzing the rate of specific metabolite formation under the different conditions.

#### Is in vitro cytochrome P450 inhibition a definitive test?

No. Properly designed and conducted in vivo drug-drug interaction studies are the definitive tests. However, in vitro tests are useful for focusing in vivo tests to areas of concern.

#### What are reversible and time-dependent inhibitors?

Reversible cytochrome P450 inhibition occurs immediately when the inhibitor is added to the cytochrome P450 enzyme. Inhibition stops when the inhibitor is removed. Time-dependent inhibitors (TDIs) exhibit increasing degrees of inhibition over the course of an incubation of the cytochrome P450, inhibitor and NADPH. TDI can occur by several mechanisms. For example, covalent modification of the cytochrome P450 by a reactive intermediate, production of a metabolite which binds tightly (but not covalently) to the cytochrome P450's active site, or production of a metabolite which is more inhibitory than the parent compound. Reversible inhibitors and TDIs require different testing protocols for effective detection.

#### What are $IC_{50}$ and $K_i$ values?

An  $IC_{50}$  value is the concentration of inhibitor which results in 50% inhibition of the cytochrome P450.  $IC_{50}$  values can be profoundly influenced by some experimental conditions such as substrate concentration. The  $K_i$  value is the inhibition constant of the free inhibitor with the cytochrome P450. In principle, it is more independent of experimental conditions. For competitive inhibitors, the  $IC_{50}$  value measured at a substrate concentration equal to the apparent  $K_m$  is 2x the  $K_i$  value.

## Do all substrates and sources of enzyme for a particular cytochrome P450 give the same results?

As mentioned above,  $IC_{50}$  values can be profoundly influenced by experimental conditions. In principle, the  $K_i$  values should be more consistent than  $IC_{50}$  values.

However, many times the  $K_i$  value is calculated based on the added (or nominal concentration) and many processes (e.g. binding to the assay matrix, adsorption to the surface of the vessel, selectivity of the probe substrate for the cytochrome P450 under the specific assay conditions and inhibitor depletion by metabolism) can have differential impact on the relationship between the nominal and free inhibitor concentrations.

## What source of enzyme should I use?

A variety of enzyme sources (e.g. cDNA-expressed, microsomal and hepatocytes) can be used. All are different.

For example, cDNA-expressed enzymes are a single enzyme (thus providing absolute specificity for the probe substrate and lower potential for inhibitor depletion) in a membrane environment derived from cultured cells and which levels of redox partners (cytochrome P450:NADPH oxidoreductase and cytochrome  $b_5$ ) which may differ from physiological levels. Because of the absolute specificity, substrates compatible with high throughput metabolite detection technologies (e.g. fluorometric) can be used. These systems are often used in the lead optimization phase or in early development.

Liver microsomes contain the cytochrome P450 in a native lipid environment with physiological levels of redox partners. The probe substrate may be selective (as opposed to specific) with other cytochromes P450 contributing to overall metabolism at some level. This contribution could vary from lot to lot. Because there are multiple cytochromes P450, there is a higher potential for inhibitor depletion. Regulatory guidance documents express a preference for the use of human liver microsomes (HLMs). This enzyme source is commonly used in drug development. Higher throughput mass spectrometry methods have also enabled using HLM-based assays in lead optimization and early development.

Hepatocytes provide the cytochrome P450 in a highly physiological environment. However, they present the cell membrane between the enzyme and added substrate plus inhibitor. This additional barrier introduces additional uncertainty in relating the nominal inhibitor concentration to the free inhibitor concentration at the enzyme. Hepatocytes are infrequently used.

## What enzymes should I test?

Methods exist for measuring the inhibition of many human cytochromes P450. The particular enzymes of highest interest will be determined by the clearance pathways of potential co-medications when the drug enters clinical use. Most often, CYP3A4 and CYP2C9 will be of concern. However, there are known drug interactions for nearly all major human hepatic cytochromes P450. In addition, there are regulatory guidance documents which provide recommendations for this testing for drug registration. Please check the current guidance documents. US-FDA Draft Guidance discussed testing for CYP1A2, 2C8, 2C9, 2C19, 2D6 and 3A. There may be specific cases where testing of CYP2B6, 2A6 and 2E1 has merit.

## What probe substrates should I use?

There are a variety of drug substrates, fluorometric substrates, luminometric substrates and spectrophotometric substrates which have been reported to be suitable for use for cytochrome P450 inhibition testing. Substrates more compatible for high throughput detection modalities (e.g. fluorometric and luminometric) are more commonly used in drug discovery and lead optimization. Drug substrates are more commonly used in drug development. The US FDA Guidance document contains a list of preferred and acceptable drug substrates for cytochrome P450 inhibition testing. BD Biosciences provides substrates, metabolite standards and heavy-labeled metabolite standards to many drug substrates as well as a number of fluorometric cytochrome P450 substrates.

## What is a heavy-labeled internal standard and why would I want to use one?

A heavy label internal standard is a metabolite standard synthesized to have several atoms (3 or more) which are heavier, stable isotopes and are typically used in mass spectrometric assays. The use of a heavy labeled standard provides a rigorous control against the possibility of ion suppression or some other artifact suppressing the levels of metabolites detected in an assay. Use of these standards is best practices. BD Biosciences provides heavy-labeled standards for metabolites for many cytochrome P450 substrates.

## Should I use a positive control?

Use of a positive control inhibitor is recommended. The table below contains some useful inhibitors:

Enzyme	Reversible Inhibition	Time-Dependent Inhibition
CYP1A2	Alpha-Naphthoflavone	Furafylline
CYP2A6	<i>Tranylcypromine</i>	<i>8-Methoxy psoralen</i>
CYP2B6	<i>Ketoconazole</i>	<i>Ticlopidine</i>
CYP2C8	<i>Montelukast</i>	<i>Gemfibrozil-glucuronide</i>
CYP2C9	<i>Sulfaphenazole</i>	<i>Tienylic Acid</i>
CYP2C19	<i>Benzyl nirvanol</i>	<i>(S)-Fluoxetine</i>
CYP2D6	<i>Quinidine</i>	<i>Paroxetine</i>
CYP2E1	<i>Chlormethiazole*</i>	<i>Diethyldithiocarbamic acid</i>
CYP3A	<i>Ketoconazole</i>	<i>Azamulin</i>



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