

CYP-MEDIATED DRUG-DRUG INTERACTION

It is not uncommon that a patient may be taking several drugs, simultaneously, for the treatment of one or more health related issues. A drug may affect or be affected by other co-administered drugs. Metabolism-based drug-drug interactions occur when a drug inhibits or induces the activity of a drug metabolizing enzyme, that catalyzes the metabolism of a concomitant drug. Metabolism-based drug-drug interactions (DDI) are one of the major factors that cause drug failures during drug development. Early stage screening of compounds for potential drug-drug interactions using *in vitro* techniques becomes necessary in order to decrease late stage compound attrition. This early *in vitro* screening of drug-drug interactions facilitates the drug discovery and development process ¹. The *in vitro* data obtained can be used to rule out the necessity of an *in vivo* study to evaluate DDI or to help guide clinical study design ². Cerep provides a wide variety of valuable metabolism-based drug-drug interaction screening assays, such as cytochrome P450 (CYP) inhibition assays and CYP induction assays.

¹ FDA Guidance for Industry (1997) - Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro

² FDA Drug Interaction Studies (2006) - Study Design, Data Analysis, and Implications for Dosing and Labeling

IN VITRO CYP INHIBITION

If a drug inhibits CYPs that catalyze metabolism of a concomitant drug, it can increase plasma concentrations of this drug, leading to potential toxicity. *In vitro* assays can adequately screen the inhibition potential of test compounds. In general, if the results are negative, a subsequent *in vivo* study may be deemed unnecessary since inhibition *in vivo* is unlikely to occur. If the results are positive, an *in vivo* study is needed to confirm the observed *in vitro* inhibition.

The high throughput fluorimetric CYP inhibition screening assay is the most rapid and cost-effective approach to test the inhibition potential. The probe substrates used in these assays are derivatives of coumarin or resorufin, which after dealkylation by CYPs will generate fluorescent products. Because these substrates are generally not highly specific to the individual CYP isozyme, they can only be used with recombinant CYP enzymes. These fluorimetric assays are often used for primary screening. Compounds that are fluorescent may interfere with the assay. In this case, one may choose the assays using drug probes with HPLC-MS detection.

A more definitive and confirmative investigation of CYP inhibition uses traditional probe substrates, which are more specific to individual CYP isoforms. For instance, midazolam 1-hydroxylation and testosterone 6 β -hydroxylation are pathways catalyzed predominantly by CYP3A4/5, dextromethorphan O-demethylation is mainly catalyzed by CYP2D6. Because these substrates are more specific to individual CYP isoforms, the inhibition assays can be performed with not only the individual recombinant CYP isoforms but also liver microsomes or other tissue preparations, which contain multiple CYP enzymes. HPLC-MS/MS methods are used to detect metabolites in these assays.

The following 26 CYP inhibition assays are currently offered at Cerep. Compounds can be tested at single concentrations (typically 10 μ M) or multiple concentrations (typically 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 μ M) for IC₅₀ determinations.

CYP	Substrate	Enzyme	Detection	Reference inhibitor
CYP1A2	CEC (5 μ M)	recombinant (1.25 pmol/ml)	fluorimetry	furafylline (IC ₅₀ : 2.7 μ M)
CYP2B6	EFC (1.5 μ M)	recombinant (10 pmol/ml)	fluorimetry	ketoconazole (IC ₅₀ : 13 μ M)
CYP2C8	DBF (0.25 μ M)	recombinant (20 pmol/ml)	fluorimetry	quercetin (IC ₅₀ : 5.1 μ M)
CYP2C9	MFC (50 μ M)	recombinant (15 pmol/ml)	fluorimetry	sulfaphenazole (IC ₅₀ : 0.2 μ M)
CYP2C19	CEC (25 μ M)	recombinant (10 pmol/ml)	fluorimetry	tranylcypromine (IC ₅₀ : 4.1 μ M)
CYP2D6	MFC (50 μ M)	recombinant (50 pmol/ml)	fluorimetry	quinidine (IC ₅₀ : 55 nM)
CYP2E1	EC (4 μ M)	recombinant (15 pmol/ml)	fluorimetry	4-methylpyrazole (IC ₅₀ : 1.9 μ M)
CYP3A4	BFC (50 μ M)	recombinant (2.5 pmol/ml)	fluorimetry	ketoconazole (IC ₅₀ : 0.8 μ M)
CYP3A4	BzRes (1 μ M)	recombinant (5 pmol/ml)	fluorimetry	ketoconazole (IC ₅₀ : 0.5 μ M)
CYP3A5	BFC (20 μ M)	recombinant (2.5 pmol/ml)	fluorimetry	ketoconazole (IC ₅₀ : 1.0 μ M)
CYP1A	phenacetin (10 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	furafylline (IC ₅₀ : 1.2 μ M)
CYP2A6	coumarin (2 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	tranylcypromine (IC ₅₀ : 0.29 μ M)
CYP2B6	bupropion (100 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	clopidogrel (IC ₅₀ : 0.2 μ M)
CYP2C8	paclitaxel (10 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	nicardipine (IC ₅₀ : 2.2 μ M)
CYP2C9	diclofenac (10 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	sulfaphenazole (IC ₅₀ : 0.68 μ M)
CYP2C19	omeprazole (0.5 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	oxybutynin (IC ₅₀ : 7.6 μ M)
CYP2D6	dextromethorphan (5 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	quinidine (IC ₅₀ : 0.13 μ M)
CYP2E1	chlorzoxazone (100 μ M)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	4-methylpyrazole (IC ₅₀ : 0.32 μ M)

Abbreviations

BFC	7-Benzyloxy-4-trifluoromethylcoumarin	EC	7-Ethoxycoumarin
BzRes	7-Benzyloxyresorufin	EFC	7-Ethoxy-4-trifluoromethylcoumarin
CEC	3-Cyano-7-ethoxycoumarin	MFC	7-Methoxy-4-trifluoromethylcoumarin
DBF	Dibenzylfluorescein		

CYP	Substrate	Enzyme	Detection	Reference inhibitor
CYP3A*	midazolam (5 µM)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	ketoconazole (IC ₅₀ : 0.3 µM)
CYP3A*	testosterone (50 µM)	liver microsomes (0.1 mg/ml)	HPLC-MS/MS	ketoconazole (IC ₅₀ : 0.3 µM)
CYP1A2	phenacetin (10 µM)	recombinant (5 pmol/ml)	HPLC-MS/MS	furafylline (IC ₅₀ : 0.8 µM)
CYP2C9	diclofenac (10 µM)	recombinant (40 pmol/ml)	HPLC-MS/MS	sulfaphenazole (IC ₅₀ : 2.5 µM)
CYP2C19	omeprazole (0.5 µM)	recombinant (1 pmol/ml)	HPLC-MS/MS	tranylcypromine (IC ₅₀ : 6.2 µM)
CYP2D6	dextromethorphan (5 µM)	recombinant (15 pmol/ml)	HPLC-MS/MS	quinidine (IC ₅₀ : 65 nM)
CYP3A4*	midazolam (5 µM)	recombinant (20 pmol/ml)	HPLC-MS/MS	ketoconazole (IC ₅₀ : 0.69 µM)
CYP3A4*	testosterone (50 µM)	recombinant (22 pmol/ml)	HPLC-MS/MS	ketoconazole (IC ₅₀ : 0.9 µM)

* FDA recommends the use of at least two structurally unrelated compounds as substrates for CYP3A4 inhibition screening.

Using traditional CYP probe substrates, CYP inhibition can also be assessed using a cocktail approach, i.e. several probe substrates are added together and incubated simultaneously with a test compound. Either human liver microsomes or a mixture of human recombinant CYP isozymes can be used as the enzyme source. Because of the substrate specificity, each CYP isozyme metabolizes only its specific corresponding probe substrate. Substrates are chosen such that none of the substrates used in this assay should be an inhibitor of any other CYP isozyme. Therefore, CYP inhibition performed using a cocktail approach can generate results comparable to those produced from the single substrate assays described above. This cocktail approach allows one incubation to assess inhibitory effects of a compound on multiple CYPs, leading to higher throughput and greater cost-effectiveness. It is thus particularly well suited for screening a large number of compounds within a short period of time. Cerep offers 2 CYP cocktail inhibition assays, one with HLM and the other with a mixture of 5 human recombinant CYP isozymes (1A2, 2C9, 2C19, 2D6 and 3A4). In both assays a mixture of 5 CYP specific substrates are included, and the respective metabolites are detected by HPLC-MS/MS analysis. Five known CYP selective inhibitors are included in each assay as reference compounds and are tested individually at multiple concentrations for IC₅₀ determinations.

Assay matrix	Substrates (as a mixture)	Protein/enzyme concentration	Reference inhibitor (tested individually)
HLM	phenacetin (10 µM) diclofenac (10 µM) omeprazole (0.5 µM) dextromethorphan (5 µM) midazolam (5 µM)	HLM(0.1 mg/ml)	furafylline sulfaphenazole tranylcypromine quinidine ketoconazole
Mixture of 5 rCYPs	phenacetin (10 µM) diclofenac (10 µM) omeprazole (0.5 µM) dextromethorphan (5 µM) midazolam (5 µM)	rCYP1A2 (1 pmol/ml) rCYP2C9 (1 pmol/ml) rCYP2C19 (1 pmol/ml) rCYP2D6 (1 pmol/ml) rCYP3A4 (1 pmol/ml)	furafylline sulfaphenazole tranylcypromine quinidine ketoconazole

The current average IC₅₀ values of the 5 reference compounds from the two cocktail CYP inhibition assays are presented below.

Isozyme inhibited	Reference Inhibitors				
	furafylline	sulfaphenazole	tranylcypromine	quinidine	ketoconazole
Assay matrix: pooled human liver microsomes					
CYP 1A	4.9	>500	24	>500	26
CYP2C9	>500	0.59	31	>500	12
CYP2C19	>500	>500	10	2.6	6
CYP2D6	>500	>500	29	0.07	27
CYP3A	>500	>500	240	59	0.07
Assay matrix: mixture of 5 human recombinant CYPs					
CYP1A2	0.9	>500	47	>500	134
CYP2C9	>500	1.1	75	>500	25
CYP2C19	254	>500	7.7	45	6.7
CYP2D6	>500	>500	42	0.02	49
CYP3A4	>500	136	398	68	0.15

TIME-DEPENDENT CYP INHIBITION

Time-dependent inhibition is also referred to as mechanism-based inhibition, which usually results from irreversible or quasi-irreversible binding of a test compound or its metabolite to the active site of a CYP enzyme during the biotransformation process. Thus, with each metabolite generated a certain portion of enzyme is inactivated. The percent inhibition increases with incubation time. Based on this mechanism, time-dependent inhibition can be identified by two sets of simultaneous experiments: one is to pre-incubate enzyme (typically for 30 min) with a test compound in the presence of the cofactor NADPH, which generates metabolites, while the other is to pre-incubate enzyme with the test compound in the absence of NADPH, which does not generate metabolites. The residual CYP enzyme activity is then analyzed by incubation of diluted pre-incubation mixture (generally 1 to 10 dilution) with a probe substrate. If the test compound is a time-dependent inhibitor, it should exhibit a higher percent inhibition (or lower IC₅₀ value) after a pre-incubation in the presence of NADPH. Conversely, if the test compound does not demonstrate an increase in inhibition after a pre-incubation in the presence of NADPH, the compound is not considered a time-dependent inhibitor. Apparently, the inhibitory potency of a time-dependent inhibitor would be underestimated in direct CYP inhibition assays (without pre-incubation of the inhibitor with the enzyme).

Both recombinant enzyme and liver microsomes can be used in time-dependent inhibition assays, however, the latter is recommended because the metabolite that inactivates a CYP isozyme can also be generated by other CYP isozymes present in liver microsomes. For this reason, a false negative result could be generated from the assay using individual recombinant CYPs.

Currently 5 time-dependent CYP inhibition assays are offered at Cerep. Pooled human liver microsomal preparation (1 mg/mL in pre-incubation and 0.1 mg/mL in final incubation with substrate) is used as the enzyme source and the respective metabolites are detected by HPLC-MS/MS methods.

CYP	Substrate	Time-dependent inhibitor	Non time-dependent inhibitor
CYP1A	phenacetin (50 µM)	furafylline IC ₅₀ = 0.95 µM for +NADPH IC ₅₀ > 10 µM for -NADPH	fluvoxamine IC ₅₀ = 0.19 µM for +NADPH IC ₅₀ = 0.41 µM for -NADPH
CYP2C9	diclofenac (50 µM)	tienilic acid IC ₅₀ = 0.59 µM for +NADPH IC ₅₀ > 10 µM for -NADPH	sulfaphenazole IC ₅₀ = 9.1 µM for +NADPH IC ₅₀ = 11 µM for -NADPH
CYP2C19	omeprazole (10 µM)	ticlopidine IC ₅₀ = 163 µM for +NADPH IC ₅₀ > 1000 µM for -NADPH	oxybutynin IC ₅₀ = 173 µM for +NADPH IC ₅₀ = 185 µM for -NADPH
CYP2D6	dextromethorphan (25 µM)	methylenedioxyamphetamine (MDMA) IC ₅₀ = 0.67 µM for +NADPH IC ₅₀ not calculable µM for -NADPH	quinidine IC ₅₀ = 2.0 µM for +NADPH IC ₅₀ = 2.7 µM for -NADPH
CYP3A	midazolam (25 µM)	troleandomycin IC ₅₀ = 1.6 µM for +NADPH IC ₅₀ not calculable for -NADPH	ketoconazole IC ₅₀ = 2.8 µM for +NADPH IC ₅₀ = 2.0 µM for -NADPH

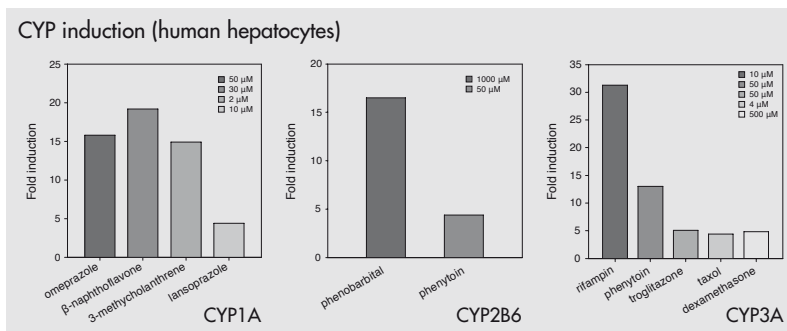
CYP INDUCTION

Some chemicals can induce the activity of CYP enzymes. These inducers will increase the metabolism of the co-administered drugs that are substrates of the induced CYP enzymes, resulting in these drugs losing efficacy. CYP enzymes such as CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 are susceptible to induction. While cellular CYP1A and CYP2B6 inductions are mediated by Aryl hydrocarbon receptor (AhR) and constitutive androstane receptor (CAR), respectively, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 are induced through pregnane X receptor (PXR). Based on the identified cellular mechanism, if a compound is not a CYP3A inducer, it will not induce these CYP2C isoforms. Cerep's *in vitro* CYP induction assays allow identification of compounds that may induce CYP activities, causing potential drug-drug interactions.

CYP	Substrate	Detected metabolite	Positive control
CYP1A	ethoxyresorufin (2 µM)	resorufin	omeprazole (50 µM)
CYP2B6	bupropion (200 µM)	hydroxybupropion	phenobarbital (1 mM)
CYP3A	midazolam (10 µM)	1-hydroxymidazolam	rifampin (10 µM)
CYP1A	ethoxyresorufin (2 µM)	resorufin	β-naphthoflavone (10 µM)
CYP3A	midazolam (10 µM)	1-hydroxymidazolam	dexamethasone (50 µM)

Cerep's induction assays are performed using plateable cryopreserved human (male or female, single donors) or rat (female, Sprague-Dawley, pool of 10 or more) hepatocytes (0.7 million cells/mL). The hepatocytes are first plated and incubated with cell culture medium for 2 days to allow recovery from processing procedures and adapt them to the culture environment. The hepatocytes are then exposed to the test compound at 3 concentrations that span the therapeutic range, including one concentration that is 10 times greater than the average expected plasma drug concentration (our default concentrations are 1, 10 and 100 µM in case the therapeutic range is unknown), in triplicate, for 2 days. At the end of the 2-day exposure, the CYP activity is determined using the respective substrate. The corresponding metabolite is detected by HPLC-MS/MS methods. The negative control (solvent only) and positive controls are included in each assay. Per FDA guidance (2006), a CYP induction study should be conducted using human hepatocytes from 3 individual donors.

The result is expressed as % of the positive control (by comparing net activity changes between test compound treated cells and positive control treated cells). A test compound is considered as an *in vitro* CYP inducer if it produces a change that is equal to or greater than 40% of the positive control, and a subsequent *in vivo* evaluation is warranted.



QUALITY CONTROL

In each assay, the respective reference compound is tested concurrently with the test compound in order to assess the assay suitability. The data are compared with historical values determined at Cerep. The assay is rendered valid if the suitability criteria are met, in accordance with the corresponding Standard Operating Procedure.

■ **QUESTIONS OR CONCERNS?**
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