Pitfalls in the design of metabolism-dependent CYP inhibition (MDI) experiments with a dilution step: Inhibitor depletion by metabolism and/or microsomal binding leads to underestimation of the shifted IC_{50} value.

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ABSTRACT

We previously demonstrated that, when a dilution step is used to assess MDI potential (*i.e.*, IC_{50} shift experiments), IC_{50} values for direct-inhibition and MDI should be processed based on the final, post-dilution concentration and the initial, pre-dilution concentration, respectively (Paris *et al.*, 2009). When processed appropriately, the "shifted IC_{50} values" (those determined following a 30-min preincubation of the drug candidate with NADPH-fortified HLM) were notably higher for several known MDIs in experiments conducted with a 10-fold dilution step than those determined by a non-dilution method, suggesting less MDI occurs at the higher concentration of HLM. More than a two-fold difference in shifted IC_{50} values was observed between the dilution and non-dilution methods for five of ten inhibitors commonly used positive controls in MDI experiments; namely ticlopidine (CYP2B6), tienillic acid (CYP2C9), paroxetine (CYP2D6), *S*-fluoxetine (CYP2C19) and azamulin (CYP3A4). Experiments were performed to determine whether these discrepancies in shifted IC_{50} values between are attributable to **1)** decreased free inhibitor concentration (fu_{inc}) and/or **2)** extensive metabolism of the inhibitor. In the case of *S*-fluoxetine, microsomal binding, not extensive metabolism, leads to the discrepancy. Furthermore, correction for microsomal binding alone resulted in shifted IC_{50} values (based on fu_{inc}) that agreed within 6% for dilution (14 → 3.1 µM) and non-dilution (5.3 → 2.9 µM) methods. Conversely, correction for observed microsomal binding alone did not resolve the discrepancies in shifted IC_{50} values for ticlopidine, tienillic acid, paroxetine and azamulin. In these cases, extensive metabolism-dependent inhibitor depletion was observed in incubations with high concentrations of HLM (up to 1 mg/mL). In the case of ticlopidine and azamulin, extensive inhibitor depletion was observed even at low concentrations of HLM (≤ 0.1 mg/mL). For these rapidly metabolized inhibitors, the IC_{50} shift could be improved not by increasing the concentration of HLM (as occurs with the dilution method) but by decreasing the concentration of HLM (≤ 0.01 mg/mL). These results establish that the non-dilution method for assessing MDI is more sensitive than the dilution method because the latter can result in substantially less CYP inactivation due to inhibitor depletion and/or a decrease in free inhibitor concentration due to microsomal binding.

Reference:
Paris BL, Kazmi F, Buckley DB, Ogilvie BW, Gipson AE and Parkinson A (2009) Pitfalls in the design of CYP inhibition studies incorporating a dilution step to examine time-dependent inhibition (TDI) or metabolism-dependent inhibition (MDI). *Drug Metab Reviews* **41**: 92-93.

XENOTECH

INTRODUCTION

Previously, we demonstrated that, when a dilution step is used to assess MDI potential (*i.e.*, IC_{50} shift experiments), IC_{50} values for direct-inhibition and MDI should be processed based on the final, post-dilution concentration and the initial, pre-dilution concentration, respectively (Paris *et al.*, 2009a). When processed appropriately, the "shifted IC_{50} values" (those determined following a 30-min preincubation of the drug candidate with NADPH-fortified HLM) were notably higher for several known MDIs in experiments conducted with a 10-fold dilution step than those determined by a non-dilution method, suggesting less MDI occurs at the higher concentration of HLM. For these inhibitors, more than a two-fold difference in shifted IC_{50} values was observed between the dilution and non-dilution methods. In these studies, it was also demonstrated that IC_{50} shifts were more pronounced in experiments conducted without a dilution step, compared to those with a dilution step, (Paris *et al.*, 2009a).

In IC_{50} shift experiments with a dilution step, preincubations are commonly performed with high concentrations of HLM (*e.g.*, ≥ 1 mg/mL) followed by a dilution step (generally 10-fold or greater) to reduce the direct inhibitory effect of the drug candidate (Van *et al.*, 2006; Van *et al.*, 2007; Venkatakrishnan *et al.*, 2007). In these preincubations with high concentrations of HLM, non-specific binding and inhibitor depletion can potentially lower the total and/or free concentrations of inhibitor (fu_{inc}). Based on these principles, it was postulated that the aforementioned discrepancies in shifted IC_{50} values between the dilution and non-dilution methods may be attributable to **1)** decreased free inhibitor concentration (fu_{inc}) and/or **2)** extensive metabolism of the inhibitor. The current study demonstrates that microsomal binding and extensive metabolism-dependent inhibitor depletion decrease the sensitivity of IC_{50} shift experiments when they are conducted with dilution methods.

RESULTS

Table 1 summarizes shifted IC_{50} values (*i.e.*, the IC_{50} value following a 30-min preincubation with NADPH-fortified HLM) from both dilution and non-dilution experiments to determine the "sensitivity" (*i.e.*, the lowest shifted IC_{50} value) of each method. IC_{50} ratios were calculated as the ratio of the shifted IC_{50} value determined without dilution divided by the shifted IC_{50} value determined with a 10-fold dilution step and the values are summarized in **Table 1**. For experiments conducted with a dilution step, IC_{50} values were calculated from data processed with inhibitor concentrations present in the initial preincubation.

- Regardless of whether IC_{50} shift experiments were conducted with or without a dilution step, IC_{50} ratios were within a factor of two (*i.e.*, an IC_{50} ratio between 0.5 and 2.0) for five of the ten inhibitors evaluated, namely furafylline (CYP1A2), gemfibrozil glucuronide (CYP2C8), diltiazem (CYP3A4), verapamil (CYP3A4) and troleandomycin (CYP3A4).
- In the case of ticlopidine (CYP2B6), tienillic acid (CYP2C9), *S*-fluoxetine (CYP2C19), paroxetine (CYP2D6) and azamulin (CYP3A4), shifted IC_{50} values differed more than two-fold between dilution and non-dilution methods. In each case, the non-dilution method was more "sensitive" (lower shifted IC_{50} value) than those conducted with the dilution method (IC_{50} ratios ranged from 0.094 to 0.38).

Figure 1 demonstrates extensive loss of selected inhibitors in preincubations with high concentrations of HLM due to metabolism. Experiments were conducted with ticlopidine, tienillic acid, *S*-fluoxetine, paroxetine and azamulin, at concentrations near their shifted IC_{50} values, to determine the extent of metabolism at several incubation times (0 – 15 min) and with multiple concentrations of NADPH-fortified HLM.

- Three inhibitors, ticlopidine, tienillic acid and azamulin, were rapidly and extensively metabolized in incubations with the highest concentrations of HLM (**Figure 1, Table 2**). In the case of ticlopidine, tienillic acid and azamulin, greater than 95% of the inhibitor was metabolized within the first five minutes of the incubation with 1.0 mg/mL HLM. In the case of paroxetine, approximately 60% loss of inhibitor was observed over the 15-min incubation at 1.0 mg/mL HLM.
- Conversely, *S*-fluoxetine was not rapidly or extensively metabolized; less than 10% loss was observed even with 1.0 mg/mL HLM.

Table 2 demonstrates that the discrepancies in shifted IC_{50} values between dilution and non-dilution methods are attributable not only to inhibitor depletion but also to a decrease in the free concentration of inhibitor (fu_{inc}) in incubations with high concentrations of HLM.

- Three of five inhibitors tested, namely, ticlopidine, *S*-fluoxetine and paroxetine, were highly bound (>75%) to microsomes at 1.0 mg/mL HLM. In each case, the extent of microsomal binding decreased in incubations containing 0.1 mg/mL HLM.
- Shifted IC_{50} values and IC_{50} ratios were corrected for observed microsomal binding. In the case of *S*-fluoxetine, correction for microsomal binding resulted in shifted IC_{50} values with less than a 10% difference between dilution and non-dilution methods (**Table 3**).
- Conversely, correction for observed microsomal binding alone did not resolve the discrepancies in shifted IC_{50} values for ticlopidine, tienillic acid, paroxetine and azamulin.

Figure 2 illustrates that ticlopidine (0.2 µM) caused 87.2% irreversible inhibition of CYP2B6 activity at 0.1 mg/mL HLM; whereas, it caused only 24.2% inhibition of CYP2B6 when preincubated with 1.0 mg/mL HLM. When preincubations were performed with 2 or 3 mg/mL HLM, ticlopidine caused less than 15% irreversible inactivation of CYP2B6 activity. Similarly, at 0.1 mg/mL HLM, *S*-fluoxetine (10 µM) caused 88.5% irreversible inhibition of CYP2C19 activity; however, it caused only 34.3% CYP2C19 inhibition when preincubated with 1.0 mg/mL HLM. Like ticlopidine and CYP2B6, *S*-fluoxetine caused little irreversible inactivation of CYP2C19 at very high concentrations of HLM.

Table 4 illustrates that lowering the concentrations of HLM improves the sensitivity of the non-dilution IC_{50} shift experiment. Whereas the IC_{50} values for direct inhibition CYP2B6 (ticlopidine), CYP2C9 (tienillic acid), CYP2D6 (paroxetine), and CYP3A4 (azamulin) remained relatively unchanged, the shifted IC_{50} values for MDI decreased in each case when the experiment was conducted at one-tenth the microsomal protein (*i.e.*, 0.01 mg/mL). Consequently, the observed IC_{50} shifts increased when assays were conducted with very low concentrations of HLM.

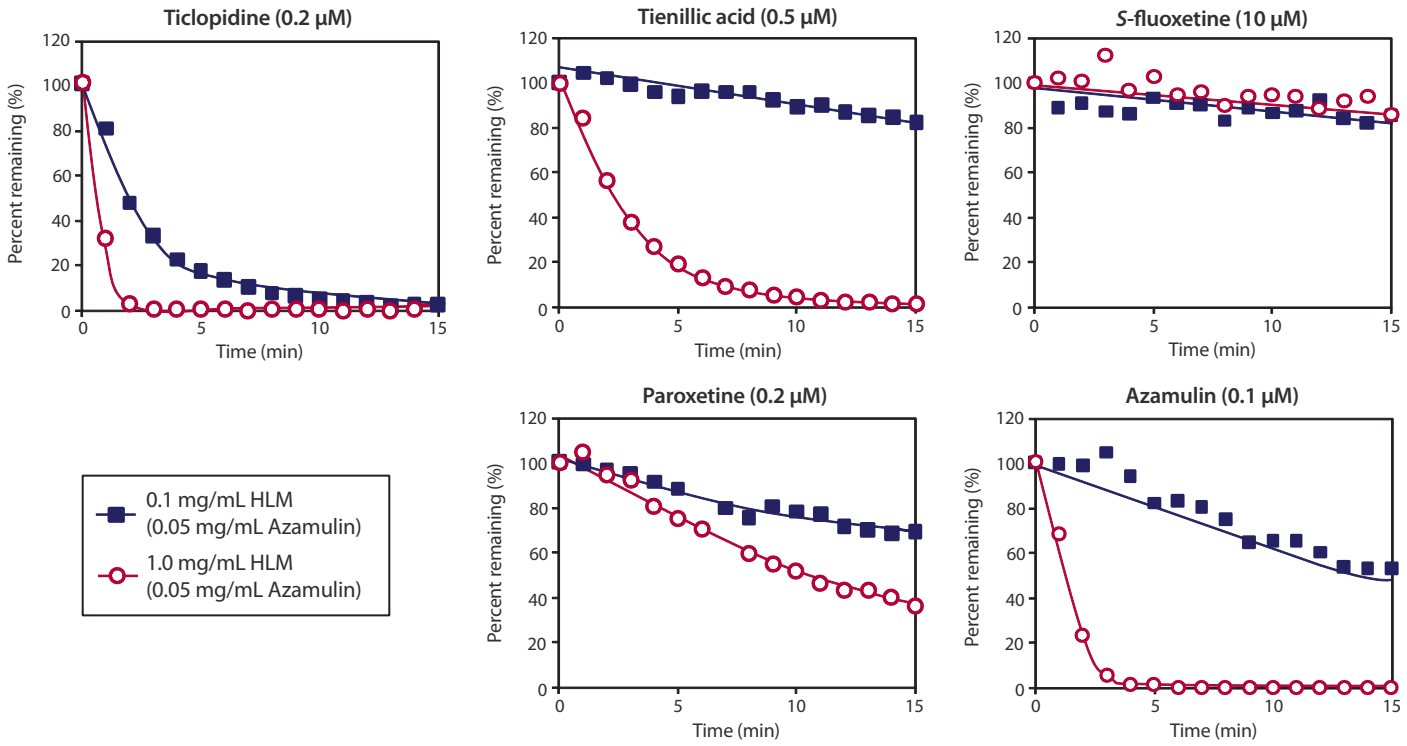
Table 1.

Shifted IC_{50} values (IC_{50} values determined after a 30-min preincubation of inhibitor with NADPH-fortified HLM) were determined with or without a 10-fold dilution step, the IC_{50} values were based on the initial (pre-dilution) concentration of inhibitor

Inhibitor	Marker Substrate	P450 Enzyme	Shifted IC_{50} (µM) (no dilution)	Shifted IC_{50} (µM) (10-fold dilution)	IC_{50} Ratio
Furafylline	phenacetin	CYP1A2	0.28	0.36	0.78
Ticlopidine	buproprion	CYP2B6	0.047	0.39	0.12
Gemfibrozil glucuronide	amodiaquine	CYP2C8	2.1	2.8	0.75
Tienillic acid	diclofenac	CYP2C9	0.066	0.57	0.12
S-fluoxetine	*S*-mephenytoin	CYP2C19	5.3	14	0.38
Paroxetine	dextromethorphan	CYP2D6	0.05	0.53	0.094
Verapamil	midazolam	CYP3A4	7.1	4.1	1.7
Diltiazem	midazolam	CYP3A4	25	24	1.0
Troleandomycin	midazolam	CYP3A4	0.85	0.63	1.3
Azamulin	midazolam	CYP3A4	0.095	0.44	0.22

Figure 1.

Time-course of inhibitor loss from incubations with NADPH-fortified HLM



CONCLUSIONS

- The data presented here demonstrate the potential shortcomings of the dilution method for the *initial* evaluation of MDIs, in part, because preincubations with high concentrations of HLM can result in **1)** extensive microsomal binding and/or **2)** inhibitor depletion due to metabolism.
- These issues can be overcome by using a non-dilution method with low concentrations of HLM. The sensitivity of the non-dilution IC_{50} shift experiment to detect MDIs can be improved by using very low concentrations of HLM as demonstrated ticlopidine and *S*-fluoxetine.
- Not only are IC_{50} shift experiments impacted by microsomal content, but K_{inact}/K_I experiments conducted with a dilution step are subject to microsomal binding, inhibitor depletion, and violation of the Michaelis-Menten principle that [inhibitor] >> [enzyme].
- Taken together, these results provide further evidence that the non-dilution method for assessing MDI is more sensitive than the dilution method because the latter can result in substantially less CYP inactivation due to inhibitor depletion and/or a decrease in free inhibitor concentration due to microsomal binding.

MATERIALS & METHODS

The sources of the reagents used in this study are described in Paris *et al.* (2009a,b). Pooled human liver microsomes (n=16, mixed gender) were prepared from non-transplantable, donated livers and characterized at the XenoTech, LLC (Lenexa, KS).

***In vitro* P450 inhibition**

Selected metabolism-dependent P450 inhibitors were evaluated after a pre-incubation (in the presence or absence of NADPH) with or without a 10-fold dilution step, as described previously (Paris *et al.*, 2009a). Briefly, metabolism-dependent inhibitors were preincubated with HLM in the presence or absence of NADPH for 30 minutes. After the preincubation period, the marker substrate (at a concentration approximately equal to its K_m) was added, and the incubation continued for 5 min to measure residual CYP activity.

When a dilution step was incorporated, the inhibitors were preincubated with a 10-fold higher concentration of microsomal protein. After the 30-min pre-incubation, aliquots of the preincubation mixtures were diluted 10-fold into the typical marker substrate incubations, which were terminated after 5 min.

Table 2.

The effects of microsomal protein concentration on the free concentration of selected metabolism-dependent inhibitors and inhibitor depletion by metabolism

			Percent Inhibitor Loss		
Inhibitor	**[HLM] (mg/ML)**	**NADPH**	**HLM binding**	**Metabolism**	**Metabolism + HLM binding**
Ticlopidine (0.2 µM)	0.1	—	29.7	—	29.7
	1.0	—	80.6	—	80.6
	0.1	+	—	98.5	99.8
	1.0	+	—	100	100
Tienillic acid (0.5 µM)	0.1	—	0	—	0
	1.0	—	11.8	—	11.8
	0.1	+	—	27.4	27.8
	1.0	+	—	99.5	99.8
S-fluoxetine (10 µM)	0.1	—	0	—	0
	1.0	—	11.8	—	11.8
	0.1	+	–	27.4	27.8
	1.0	+	–	99.5	99.8
Paroxetine (0.2 µM)	0.1	—	55.2	—	55.2
	1.0	—	85.8	—	85.8
	0.1	+	—	53.0	73.6
	1.0	+	—	88.0	97.6
Azamulin (0.1 µM)	0.05	—	2.5	—	2.5
	0.5	—	24.4	—	24.4
	0.05	+	—	11.0	11.0
	0.5	+	—	99.2	99.8

Time course of inhibitor metabolism

Depletion of ticlopidine, tienillic acid, *S*-fluoxetine, paroxetine and azamulin from preincubations due to microsomal metabolism was determined with incubations (2 mL, single-vessel) containing pooled HLM (typically 0.1 or 1.0 mg/mL). To determine total inhibitor concentration remaining over time, aliquots (100 µL) were removed at 1 min intervals for 15 min and mixed with an equal volume of acetonitrile.

Table 3.

Shifted IC_{50} values and IC_{50} ratios corrected for experimentally determined microsomal binding

	Uncorrected ([I] total)			Corrected (fu_{inc})		
Inhibitor	IC_{50} no dilution	IC_{50} 10-fold dilution	IC_{50} Ratio	IC_{50} no dilution	IC_{50} 10-fold dilution	IC_{50} Ratio
Ticlopidine	0.047	0.39	0.12	0.033	0.076	0.43
Tienillic acid	0.066	0.57	0.12	0.066	0.50	0.13
S-fluoxetine	5.3	14	0.38	2.9	3.1	0.94
Paroxetine	0.05	0.53	0.094	0.022	0.075	0.29
Azamulin	0.038	0.31	0.12	0.037	0.23	0.16

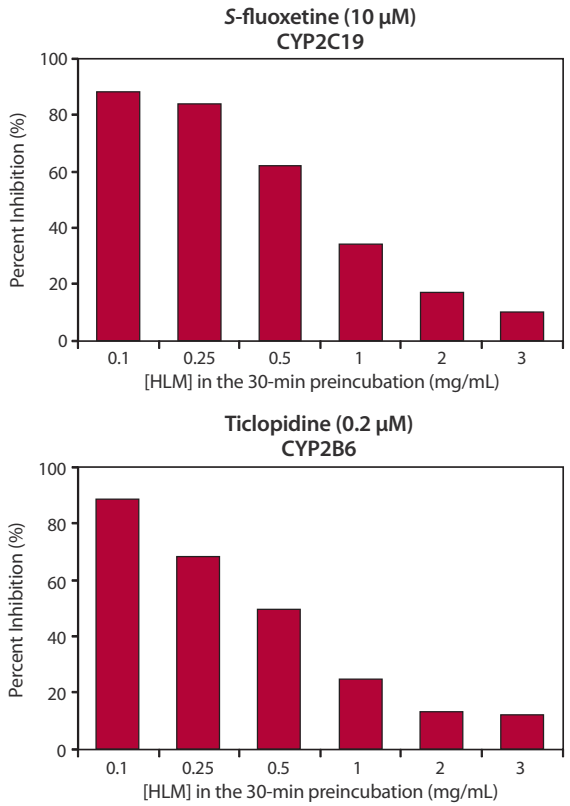
Table 4.

Decreasing microsomal protein decreases the shifted IC_{50} values and increases the magnitude of the IC_{50} shifts for extensively metabolized MDIs

Inhibitor	CYP Enzyme	[HLM] (mg/mL)	IC_{50} (μM) - NADPH	IC_{50} (μM) + NADPH	IC_{50} shift
Ticlopidine	CYP2B6	0.1	0.18	0.047	3.8
		0.01	0.22	0.011	20
Tienillic acid	CYP2C9	0.1	1.0	0.066	15
		0.01	0.52	0.013	40
Paroxetine	CYP2D6	0.1	0.73	0.051	15
		0.01	0.88	0.025	35
Azamulin	CYP3A4	0.05	0.21	0.038	5.5
		0.005	0.33	0.013	25

Figure 2.

Irreversible inhibition of CYP2C19 and CYP2B6 following preincubations with increasing concentrations of HLM



Contribution of microsomal binding and metabolism to depletion of inhibitors

The binding of ticlopidine, tienillic acid, *S*-fluoxetine, paroxetine and azamulin to HLM was determined with incubations (30 min) conducted with incubations containing pooled HLM at 37°C with or without an NADPH-generating system. Following the 30-min incubation, samples were centrifuged at approximately 21,000 RCF for 20 min at room temperature to sediment the microsomal protein. An aliquot of the supernatant was added to stop reagent and analyzed for remaining inhibitor to determine the contribution of both HLM binding and metabolism to inhibitor depletion (samples incubated with HLM in the presence of NADPH). To a second sample, stop reagent was added directly to the entire sample (containing both supernatant and HLM fractions) and analyzed for remaining inhibitor to determine the contribution of metabolism to inhibitor depletion. Third, an aliquot from the supernatant fraction from an incubation without NADPH (therefore, no P450 metabolism) was assessed for inhibitor loss due to HLM binding (fu_{inc}).

The extent of irreversible P450 inhibition in preincubations with increasing HLM concentrations

Incubations (30 min at 37°C) were performed with ticlopidine (0.2 μM) or *S*-fluoxetine (10 μM) with various concentrations of NADPH-fortified pooled HLM (0.1, 0.25, 0.5, 1, 2 and 3 mg/mL). Following a 30-min incubation, microsomal protein was re-isolated by ultracentrifugation (100,000 *x g* for 60 min at 4°C). The supernatant fraction was discarded and the resultant microsomal pellets were rinsed twice. Microsomal pellets were resuspended in 250 mM sucrose and microsomal protein concentration was determined by the Pierce BCA Protein Assay. CYP activity was assessed with marker substrate reactions at 0.1 mg/mL HLM as described above.

Analytical methods

Metabolite formation from P450 marker substrate reactions was determined by LC/MS/MS as previously described (Ogilvie *et al.*, 2008; Paris *et al.*, 2009). Analyses of inhibitor depletion were determined by LC/MS/MS.

Statistical analyses

IC_{50} values were determined by nonlinear regression with XLfit3 (version 3.0.5; ID Business Solutions Ltd., Guildford, Surrey, UK) or by GraFit (version 4.0.21; Erithricus Software Ltd., Horley, Surrey, UK).

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