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An evaluation of the dilution method for identifying metabolism-dependent inhibitors (MDIs) of cytochrome P450 (CYP) enzymes

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Abbreviations used are: CYP, Cytochrome P450; DDI, drug-drug interaction; FDA, US Food and Drug Administration; HLM, human liver microsomes; IC_{50}, inhibitor concentration that causes 50% inhibition; LC/MS/MS, liquid chromatography/tandem mass spectrometry; K_I, inhibitor concentration that causes half the maximal rate of enzyme inactivation; k_{inact}, maximal rate of enzyme inactivation; K_m, Substrate concentration supporting half the maximum rate of an enzyme-catalyzed reaction; MBI, mechanism-based inhibition; MDI, metabolism-dependent inhibition; 8-MOPS, 8-methoxypsoralen; PhRMA, Pharmaceutical Research and Manufacturers of America; TDI, time-dependent inhibition.

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ABSTRACT

Metabolism-dependent inhibition (MDI) of cytochrome P450 is usually assessed *in vitro* by examining whether the inhibitory potency of a drug candidate increases following a 30-min incubation with human liver microsomes (HLM). To augment the IC_{50} shift, many researchers incorporate a dilution step whereby the samples, after being pre-incubated for 30 min with a high concentration of HLM (with and without NADPH), are diluted prior to measuring P450 activity. In the present study, we show that the greater IC_{50} shift associated with the dilution method is a consequence of data processing. With the dilution method, IC_{50} values for direct-acting inhibitors vary with the dilution factor unless they are based on the final (post-dilution) inhibitor concentration whereas the IC_{50} values for MDIs vary with the dilution factor unless they are based on the initial (pre-dilution) concentration. When the latter data are processed on the final inhibitor concentration, as is commonly done, the IC_{50} values for MDI (shifted IC_{50} values) decrease by the magnitude of the dilution factor. The lower shifted IC_{50} values are a consequence of data processing, *not* enhanced P450 inactivation. In fact, for many MDIs, increasing the concentration of HLM actually leads to considerably *less* P450 inactivation because of inhibitor depletion and/or binding of the inhibitor to microsomes. A true increase in P450 inactivation and IC_{50} shift can be achieved by assessing MDI by a non-dilution method and by *decreasing* the concentration of HLM. These results have consequences for the conduct of MDI studies and the development of cutoff criteria.

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INTRODUCTION

Inhibition of cytochrome P450 (CYP) enzymes is a well-recognized cause of drug-drug interactions. This occurs by two general mechanisms: direct inhibition and metabolism-dependent inhibition (MDI). Both can cause clinically significant cytochrome P450 inhibition. For this reason, the US Food & Drug Administration (FDA) and the European Medical Agency (EMA) both require an *in vitro* assessment of the ability of drug candidates to cause direct inhibition and MDI of the seven major drug-metabolizing P450 enzymes in human liver microsomes (HLM) (Draft FDA Guidance for Industry, 2006; http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm080499.htm; Huang et al., 2008; EMA Guideline on the Investigation of Drug Interactions, 2010; http://www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500090112).

A recent PhRMA publication on cytochrome P450 inhibition focuses on the conduct of *in vitro* experiments to identify drug candidates that cause MDI (Grimm et al., 2009). The PhRMA consensus paper recommends that MDI be assessed *in vitro* by examining whether a 30-min incubation of the drug candidate with HLM in the presence of NADPH increases its inhibitory potency relative to a 30-min incubation in the absence of NADPH. According to the consensus paper, most pharmaceutical researchers use the fold difference between these two values (i.e., the magnitude of the IC_{50} shift) to decide whether a drug candidate should be evaluated *in vivo* for its ability to cause clinically significant MDI of cytochrome P450. Surprisingly, industry cutoff values for what constitutes evidence for MDI based on the magnitude of the IC_{50} shift range from 1.2 to 10 (Grimm et al., 2009). Furthermore, the consensus paper reported that about half of the pharmaceutical researchers surveyed incorporate a dilution step in the design of their experiments to evaluate MDI, such that, after being incubated for 30 min with a relatively high

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concentration of HLM, the samples are diluted (*e.g.,* 10 fold) prior to measuring P450 enzyme activity

The studies described here were prompted by the observation that the magnitude of the IC_{50} shifts reported with the dilution method were much greater than the magnitude of the IC_{50} shift we determined with the non-dilution method. For example, using a non-dilution method (with HLM at 0.1 mg/mL), we determined that furafylline, an irreversible MDI of CYP1A2, causes a 20-fold shift in IC_{50} value (Yerino et al., 2007), whereas a 400-fold shift was reported for the dilution method (with an initial concentration of HLM at 2.0 mg/mL followed by a 10-fold dilution to 0.2 mg/mL) (Perloff et al., 2009). This phenomenon was not unique to furafylline; all MDIs are reported to have lower shifted IC_{50} values when evaluated by the dilution method compared with the non-dilution method, which implies that all MDIs cause more enzyme inactivation when they are incubated with relatively high concentrations of HLM.

The possibility that all MDIs cause more cytochrome P450 inactivation when the concentration of HLM is increased is not only counterintuitive but it is inconsistent with the results of our studies on the effects of protein concentration on P450 inactivation by MDIs like 8-methoxypsoralen (8-MOPS) and furafylline (Ogilvie et al., 2008; Yerino et al., 2007). Accordingly, we sought another explanation for the observation that MDIs have lower shifted IC_{50} values when assessed by the dilution method. The hypothesis we developed is that the lower shifted IC_{50} values associated with the dilution method are a consequence of how the data are processed. The basis for this hypothesis is illustrated in Fig. 1, which shows the design of a typical dilution assay in which the initial concentration of HLM is 1.0 mg/mL and the final concentration, after a 10-fold dilution, is 0.1 mg/mL. The figure shows the conditions for examining direct inhibition either by performing a zero-time pre-incubation (IC_{50} curve **A**) or by incubating the inhibitor with HLM for 30 min in the absence of NADPH (IC_{50} curve **B**) as well as the conditions for examining MDI, which involves incubating the inhibitor with HLM for 30 min in

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the presence of NADPH (IC_{50} curve **C**). In this scheme, we posit that the IC_{50} value for direct inhibition (IC_{50} curves **A** and **B**) are governed by the final (post-dilution) concentration of inhibitor because direct inhibition can only occur in the presence of substrate, which is added *after* the dilution step. Conversely, we posit that the IC_{50} value for MDI (IC_{50} curve **C**) is governed by the initial (pre-dilution) concentration of inhibitor because MDI occurs during the 30-min pre-incubation of the inhibitor with HLM, which occurs *before* the dilution step. If the shifted IC_{50} values are based on the final concentration, as they commonly are, they will be artificially lowered by the dilution factor and, correspondingly, the magnitude of the IC_{50} shift will be artificially increased by a factor of 10.

We present here experimental evidence to support the hypothesis that the apparent greater sensitivity of the dilution method over the non-dilution method is a consequence of data processing. We further show that the dilution method does *not* result in more MDI of cytochrome P450 but, in many cases, actually results in considerably *less* MDI. In fact, a true increase in cytochrome P450 inactivation and an increase in the IC_{50} shift can be achieved by *decreasing* the concentration of HLM. These results have important consequences for the conduct of MDI studies and the development of cutoff criteria for MDI.

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MATERIALS AND METHODS

Chemicals and Reagents. Diltiazem, erythromycin, *S*-fluoxetine, furafylline, methimazole, mibefradil, mifepristone, paroxetine, ticlopidine, troleandomycin and verapamil were purchased from Sigma-Aldrich (St. Louis, MO); fluconazole and gemfibrozil glucuronide were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada); tienilic acid was purchased from Cypex (Dundee, Scotland, UK); azamulin was purchased from BD Biosciences (Bedford, MA). The sources of the other reagents used in this study have been described elsewhere (Robertson et al., 2000; Ogilvie et al., 2006; Paris et al., 2009).

Test system. Pooled human liver microsomes (n=16, mixed gender) were prepared from non-transplantable livers and characterized at XenoTech, LLC (Lenexa, KS) as described previously (Pearce et al., 1996; Parkinson et al., 2004).

In vitro P450 Inhibition. The effects of known P450 inhibitors were evaluated in IC_{50} shift experiments with and without a pre-incubation step (in the presence and absence of NADPH) as previously described (Ogilvie et al., 2008; Paris et al., 2009), either with no dilution or with a 10- to 40-fold dilution step. Conditions for the non-dilution method are summarized in Table 1. The inhibitors were incubated at 37°C in 200-µL incubation mixtures containing pooled HLM (≤ 0.1 mg/mL), potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4), an NADPH-generating system (consisting of 1 mM NADP, 5 mM glucose-6-phosphate and 1 Unit/ml glucose-6-phosphate dehydrogenase) and P450 marker substrate at a concentration approximately equal to its K_m, at the final concentrations indicated in Table 1. Reactions were initiated by the addition of an NADPH-generating system and terminated after 5 min by the addition of 200 µL acetonitrile containing the appropriate internal standard (deuterium-labeled metabolite). Precipitated protein was removed by centrifugation (920 × g for

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10 min at 10°C). Calibration and quality control (QC) metabolite standards were prepared in zero-time incubations. The analytical procedures are summarized in Table 1. Three IC_{50} curves were generated: (**A**) a zero-time pre-incubation with inhibitor to measure direct inhibition; (**B**) a 30-min incubation of the inhibitor with HLM in the absence of NADPH (a second measure of direct inhibition), and (**C**) a 30-min incubation of the inhibitor with HLM in the presence of NADPH to measure MDI. Selected inhibitors were also assessed with non-dilution IC_{50} shift experiments conducted at one-tenth ($1/10^{th}$) the standard concentration of HLM listed in Table 1.

When the dilution method was used, the initial concentration of HLM was increased 10-fold above the final concentration of HLM listed in Table 1. The inhibitors were pre-incubated at 37 ± 1 °C for zero minutes (**A**), for 30 min in the absence of NADPH (**B**) for 30 min in the presence of NADPH or (**C**), after which the samples were diluted 10-fold, mixed with P450 marker substrate (at the final concentrations listed in Table 1) and incubated for 5 min. Reactions were terminated by the addition of an equal volume of acetonitrile containing the appropriate internal standard and analyzed by LC/MS/MS (Table 1). In selected cases, the initial concentration of HLM was increased 20-, 30- or 40-fold and, after the 30-minute pre-incubation, the samples were diluted 20-, 30- and 40-fold, respectively.

Additional P450 inhibition experiments were conducted with selected inhibitors to determine IC_{50} values for direct inhibition (IC_{50} curve **B**) under standard conditions (detailed in Table 1) and at one-fourth the HLM concentration (*e.g.* 0.1 mg/mL → 0.025 mg/mL) but at four-times the substrate incubation time (20 min) to keep the overall extent of substrate metabolism the same.

***K_I* and *k_{inact}* determination.** To determine K_I and k_{inact} for the inactivation of CYP2C19, various concentrations of *S*-fluoxetine (3, 10, 30, 60 and 100 μM) were incubated at 37°C for 3, 6, 9, 15 or 30 min with two concentrations of NADPH-fortified pooled HLM (1.0 and 4.0 mg/mL). After

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the pre-incubation step, duplicate samples were diluted 10- or 40-fold (to give a final concentration of 0.1 mg protein/mL) into incubation medium containing 400 µM *S*-mephenytoin (~10x K_m) and an NADPH-generating system. The diluted samples were incubated for 5 min and processed to measure residual CYP2C19 activity as summarized in Table 1.

Metabolic stability of selected inhibitors. The metabolic stability of selected P450 inhibitors, namely ticlopidine (0.2 µM), tienilic acid (0.5 µM), *S*-fluoxetine (10 µM), paroxetine (0.2 µM), azamulin (0.1 µM) and mibefradil (0.2 µM), was determined at two concentrations of microsomal protein (typically 0.1 or 1.0 mg/mL, unless otherwise noted) under conditions described above for P450 inhibition experiments. To determine inhibitor loss over time, aliquots (100 µL) from a single-vessel incubation were transferred at one-min intervals for 15 min to an equal volume of acetonitrile containing an internal standard. The samples were analyzed by LC/MS/MS, as summarized in Table 2.

Microsomal binding of selected inhibitors. Selected P450 inhibitors, namely ticlopidine (0.2 µM), tienilic acid (0.5 µM), *S*-fluoxetine (10 µM), paroxetine (0.2 µM), azamulin (0.1 µM) and mibefradil (0.2 µM), were incubated (500 µL) with pooled HLM (typically 0.1 or 1.0 mg/mL) in the presence or absence of an NADPH-generating system at 37°C for 30 min. Following the 30-min incubation, microsomes were isolated in 20 min by centrifugation at ~21,000 RCF in a 5417R Eppendorf microcentrifuge at room temperature. Identical incubations without HLM served as a control for non-specific binding of the inhibitors to the incubation vessel. Following centrifugation, the samples were processed three ways. First, to determine the contribution of both non-specific binding and metabolism to inhibitor depletion, an aliquot (200 µL) of those samples that were incubated with HLM in the presence of NADPH was transferred to an equal volume of acetonitrile and analyzed by LC/MS/MS as summarized in Table 2. Second, to determine the contribution of microsomal binding alone (fu_{mic}) to inhibitor depletion, an aliquot

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(200 µL) of those samples that were incubated with HLM in the absence of NADPH (to prevent metabolic loss) was transferred to an equal volume of acetonitrile and analyzed by LC/MS/MS (Table 2). Third, to determine the contribution of metabolism alone to inhibitor depletion, an equal volume of acetonitrile was added directly to post-centrifugation samples that had been incubated with HLM in the presence of NADPH. This solubilized any inhibitor bound non-specifically to microsomes or the vessel. The amount of inhibitor remaining was determined by LC/MS/MS (Table 2).

In the case of fluoxetine, the unbound fraction at various concentrations of HLM (fu_{mic}) was estimated both experimentally (as described above) and theoretically as described by Hallifax and Houston (2006) and Austin *et al.* (2002); equations (1) and (2), respectively.

(1) $$fu_{mic} = \frac{1}{c \times 10^{0.072 \times logP/D^2 + 0.067 \times logP/D - 1.126} + 1}$$

(2) $$fu_{mic} = \frac{1}{c \times 10^{0.56 \times logP/D - 1.41} + 1}$$

Assessment of MDI reversibility. Selected P450 inhibitors, namely ticlopidine (0.2 µM), tienilic acid (0.5 µM), *S*-fluoxetine (10 µM), paroxetine (0.2 µM), azamulin (0.1 µM) and mibefradil (0.2 µM), were incubated with various concentrations of NADPH-fortified pooled HLM (0.1, 0.25, 0.5, 1, 2 and 3 mg/mL) at 37°C for 30 min under conditions described above. Incubations with solvent alone (acetonitrile; 0.5%, v/v) were conducted at each concentration of HLM and served as negative controls. Following the 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 with wash buffer (150 mM potassium chloride and 10 mM EDTA, pH 7.4) to remove residual inhibitor and/or any reversible inhibitory metabolites. The microsomal pellets were resuspended in 250 mM sucrose and microsomal protein concentration was determined by the Pierce BCA Protein Assay (Pierce,

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Rockford, IL). Residual P450 activity was assessed at a final concentration of 0.05 or 0.1 mg protein/mL as described in Table 1.

Data processing and statistical analysis. All 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), as detailed previously (Paris et al., 2009). Data from the K_I and k_{inact} determinations were processed with a validated LIMS (Galileo version 3.3; ThermoFisher Scientific, Inc.). To determine the rate of enzyme inactivation at each inhibitor concentration tested, the data were analyzed by a two-step method incorporating nonlinear regression. The first step calculated the apparent slope of enzyme inactivation (k_{obs}) for each inhibitor concentration based on the following formula (equation 3):

$$(3)\ ln\left(\frac{E_t}{E_0}\right) = -k_{obs} \times t$$

For this equation, based in part on a method described by Kitz and Wilson (1962), the natural log of the ratio of the residual activity (E_t) to the control activity (E_0) (where the residual activity is the rate after a defined preincubation period with the test article) is plotted against preincubation time for each concentration of inhibitor.

In the second step, K_I and k_{inact} were calculated by solving the non-linear equation (equation 4) described by Jones et al. (1999):

$$(4)\ \boldsymbol{k_{obs}} = \frac{(\boldsymbol{k_{inact}} \times [\boldsymbol{I}])}{(\boldsymbol{K_I} + [\boldsymbol{I}])}$$

This equation is analogous to the Michaelis-Menten equation where k_{obs} represents the rate of enzyme inactivation at each inhibitor concentration, [I] is the initial (pre-dilution) inhibitor concentration, K_I is the inhibitor concentration that produces half the maximum rate of enzyme inactivation (analogous to K_m) and k_{inact} represents the maximum rate of enzyme inactivation

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(analogous to V_{max}). This equation assumes there is negligible change in inhibitor concentration during the incubation period and that the loss of enzyme activity is due solely to enzyme inactivation.

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RESULTS

Most of the P450 inhibition experiments described below involved measuring three IC_{50} curves (designated **A**, **B** and **C** in Fig. 1). Regardless of whether the assessment of P450 inhibition was based on the non-dilution or dilution method, IC_{50} curve **A** (no pre-incubation) and IC_{50} curve **B** (a 30-min pre-incubation in the absence of NADPH) represent measures of direct inhibition whereas IC_{50} curve **C** (a 30-min pre-incubation in the presence of NADPH) represents a measure of MDI. For all the inhibitors used in this study we observed only negligible differences between IC_{50} curves **A** and **B**. For simplicity we present below only the results for IC_{50} curve **B** (as the measure of direct inhibition) and IC_{50} curve **C** (as the measure of MDI). For simplicity we also describe the concentration of HLM in the standard non-dilution method as 0.1 mg/mL and the concentration of HLM in the pre-incubation phase of the dilution method as 1.0 mg/mL even though, as shown in Table 1, the concentrations of HLM for assays with midazolam (CYP3A4) and amodiaquine (CYP2C8) were half and one-eighth the so-called standard concentration, respectively.

Data processing for direct-acting inhibitors

We hypothesized that, when P450 inhibition is assessed by the dilution method, the IC_{50} value for direct inhibition should be based on the final (post-dilution) concentration of inhibitor because direct inhibition can occur only in the presence of substrate, which is added *after* the dilution step. This hypothesis was tested with fluconazole, a direct-acting inhibitor of CYP3A4 (Tran et al., 2002), and the results are shown in Table 3 and Fig. 2 (left panels). The upper-left panel of Fig. 2 shows the results of a non-dilution experiment, in which fluconazole was pre-incubated with HLM (0.05 mg/mL) and NADPH for zero or 30 min prior to the addition of midazolam to measure CYP3A4 activity. Having confirmed that fluconazole is a direct-acting acting inhibitor of CYP3A4 with an IC_{50} value of ~6 μM (with no shift in IC_{50}), we evaluated the inhibition of CYP3A4 by fluconazole with the dilution method, which incorporated a 10-, 20-, 30- or 40-fold dilution step. The IC_{50} data were processed based on both the initial and final

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concentration of fluconazole. As predicted, when the data were processed based on the final concentration, fluconazole inhibited CYP3A4 with an IC_{50} value of 5-8 μM, which matched that determined by the non-dilution method (Table 3; Fig. 2, bottom-left panel). In contrast, when the data were processed based on the initial concentration of fluconazole, the IC_{50} values increased as the fold dilution increased (Table 3; Fig. 2, left middle panel).

Data processing for metabolism-dependent inhibition

The experiment conducted with the direct-acting inhibitor fluconazole was repeated with two MDIs of CYP3A4, namely methimazole and diltiazem. Fig. 2 shows the results obtained with the non-dilution method for methimazole (upper middle panel) and diltiazem (upper right panel). As expected, pre-incubating methimazole and diltiazem with HLM (0.05 mg/mL) for 30 min in the presence of NADPH increased their inhibitory potency towards CYP3A4 as evidenced by the leftward shift in IC_{50} values (570 → 80 μM for methimazole; 89 → 18 μM for diltiazem).

To test the hypothesis that, when cytochrome P450 inhibition is assessed by the dilution method, the IC_{50} value for MDI (IC_{50} curve **C**) should be based on the initial (pre-dilution) concentration of inhibitor (Fig. 1), we evaluated the MDI of CYP3A4 by methimazole and diltiazem with the dilution method, which incorporated a 10-, 20-, 30- or 40-fold dilution. . As predicted, when the data for MDI (IC_{50} curve **C**) were processed based on the initial inhibitor concentration, methimazole and diltiazem inhibited CYP3A4 with an IC_{50} value that closely matched (generally within a factor of two) the IC_{50} value determined by the non-dilution method (Table 3; Fig. 2). In contrast, when the data were processed based on the final inhibitor concentration (as is commonly done), the IC_{50} values decreased as the fold dilution increased. Perloff et al. (2009) and Obach et al. (2007) previously evaluated diltiazem as an MDI of CYP3A4 with an experimental design that incorporated a 10-fold dilution step and in which CYP3A4 activity was measured with both midazolam and testosterone. We performed the same 10-fold dilution experiment with both substrates and processed the data for MDI (IC_{50}

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curve **C**) from all three research groups based on the final concentration of diltiazem (like Perloff et al. and Obach et al.) and the initial concentration of diltiazem (which was shown in Table 3 and Fig. 2 to be the appropriate method for MDI). We also performed the MDI experiment by the non-dilution method. The results are shown in Fig. 3.

Panel A of Fig. 3 shows that the IC_{50} values for direct inhibition (IC_{50} curve **B**) were similar between the non-dilution and dilution method and were similar among all three groups, all of which appropriately based the values for IC_{50} curve **B** on the final concentration of diltiazem. The values for IC_{50} curve **B** reported by Obach et al. (2007) were slightly lower than those determined by Perloff et al. (2009) and us, which will be discussed later. Panel B of Fig. 3 shows that the IC_{50} values for MDI determined by the non-dilution method were roughly 10-times greater than those determined by the dilution method when the data were processed based on the final concentration of diltiazem. This 10-fold difference is also apparent from the data reported by Perloff et al. (2009) and Obach et al. (2007). Panel C of Fig. 3 shows that this 10-fold difference between the non-dilution and dilution method essentially disappeared when the data for MDI determined by the dilution method were processed based on the initial concentration of diltiazem. When the magnitude of the IC_{50} shift was calculated (i.e., the ratio of the IC_{50} value for direct inhibition and the IC_{50} value for MDI), the same trend was observed, as shown in the lower panels of Fig. 3. The magnitude of the IC_{50} shift determined by the dilution method (by all three groups) was roughly 10-times greater than that for the non-dilution method when the data for MDI were processed based on the final concentration of diltiazem (panel A/B in Fig. 3) but this difference largely disappeared when the data for MDI were processed based on the initial concentration of diltiazem (panel A/C in Fig. 3).

Dilution versus non-dilution for multiple MDIs and P450 enzymes

Table 4 shows the results of experiments where 14 known MDIs were evaluated by the non-dilution and dilution method as inhibitors of the seven major P450 enzymes (namely CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4). When the dilution method was used (with a 10-

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fold dilution in all cases), the data for direct inhibition (IC_{50} curve **B**) were processed based only on the final inhibitor concentration. However, the data for MDI (IC_{50} curve **C**) were processed based on both the final inhibitor concentration (as is commonly done) and on the initial concentration. Table 4 also contains corresponding data from comprehensive and well-documented studies by two other research groups, namely those by Perloff et al. (2009) and Obach et al. (2007). These two groups evaluated the same cytochrome P450 enzymes with many of the same MDIs and many of the same P450 substrates as were used in the current study. Both groups used the dilution method, which in most, but not all, cases involved a 10-fold dilution step. Both groups reported IC_{50} values for direct and MDI based on the final concentration of inhibitor. In Table 4, we added an additional column to the data from Perloff et al. (2009) and Obach et al. (2007) to show the IC_{50} values for MDI based on the initial concentration of inhibitor.

Table 4 shows that the IC_{50} values for direct inhibition (IC_{50} curve **B**) determined by the non-dilution method agreed well (generally within a factor of 2) with those determined by the dilution method, and they also agreed well (generally within a factor of 2) with the IC_{50} values for direct inhibition reported by Perloff et al. (2009). Without exception, the IC_{50} values for direct inhibition reported by Obach et al. (2007) were slightly lower than those determined in the current study and those reported by Perloff et al. (2009), and an explanation for this small but systematic difference is provided later. Table 4 also shows that, when the dilution method is used, the IC_{50} values for MDI (IC_{50} curve **C**) determined in the current study agreed reasonably well (generally within a factor of 2) with those determined by Perloff et al. (2009) and Obach et al. (2007), although the latter values were once again uniformly lower (with one exception).

Shifted IC_{50} values: dilution versus non-dilution

Fig. 4 compares the shifted IC_{50} curves (IC_{50} curve **C**) determined by the non-dilution method with those determined by the dilution method for 11 MDIs. In the latter case, the data were processed based on the initial inhibitor concentration (which is the appropriate

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concentration for MDI). The MDIs in Fig. 4 are divided into two groups. Those shown on the left include furafylline (CYP1A2), gemfibrozil glucuronide (CYP2C8) and three MDIs of CYP3A4, namely diltiazem, troleandomycin and verapamil. In all of these cases, the two shifted IC_{50} curves (IC_{50} curve **C** from the dilution assay and IC_{50} curve **C** from the non-dilution assay) were essentially superimposable, suggesting that the degree of P450 inactivation that occurred in the dilution and non-dilution assay was similar. The MDIs shown on the right-hand side of Fig. 4 include ticlopidine (CYP2B6), tienilic acid (CYP2C9), *S*-fluoxetine (CYP2C19), paroxetine (CYP2D6) and azamulin (CYP3A4) and mibefradil (CYP3A4). In these cases, the two shifted IC_{50} curves were not superimposable and in all cases IC_{50} curve **C** determined by the dilution method was shifted to the right of that determined by the non-dilution method, suggesting that less P450 inactivation occurred with the dilution method.

For all the MDIs shown in the left-hand panel of Fig. 4, the ratio of the two shifted IC_{50} curves (i.e., the shifted IC_{50} value for the non-dilution method divided by the shifted IC_{50} value for the dilution method) was close to unity, as illustrated in Fig. 5. Assuming values that agreed within a factor of two can be considered the same, the results in Fig. 5 suggest that furafylline (CYP1A2), gemfibrozil glucuronide (CYP2C8) and several MDIs of CYP3A4, namely verapamil, diltiazem, troleandomycin, methimazole and erythromycin, caused the same degree of P450 inactivation regardless of whether the dilution or non-dilution method was used. In contrast, for all the MDIs shown in the right-hand panel of Fig. 4, the ratio of the two shifted IC_{50} curves was less than unity and the values fell outside of the twofold range (Fig. 5). These results suggest that those MDIs shown in the right-hand panel of Fig. 4 (i.e., those MDIs with a ratio of less than 0.5 in Fig. 5) caused less P450 inactivation when assayed by the dilution method, which suggests they caused less P450 inactivation when incubated with a relatively high concentration of HLM.

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The effect of protein concentration on the extent of P450 inactivation

To investigate why the dilution method can lead to less P450 inactivation, we examined the effects of protein concentration on the extent of P450 inactivation by ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil based on an ultracentrifugation method (Buckley et al., 2008). Ticlopidine (0.2 μM), tienilic acid (0.5 μM), *S*-fluoxetine (10 μM), paroxetine (0.2 μM), azamulin (0.1 μM) and mibefradil (0.2 μM) were incubated with six concentrations of HLM (from 0.1 to 3 mg/mL) at concentrations of inhibitor known to cause extensive, irreversible P450 inactivation when incubated with HLM at 0.1 mg/mL. After a 30-min incubation in the presence of NADPH, the microsomes were isolated by ultracentrifugation, rinsed, resuspended and assayed for cytochrome P450 activity. The results are shown in Fig. 6. All six MDIs showed the same trend; as the concentration of HLM increased, the extent of P450 inactivation progressively decreased. Mibefradil (0.2 μM), for example, caused ~70% inactivation of CYP3A4 when incubated with HLM at 0.1 mg/mL, but caused less than 10% inactivation at 1.0 mg/mL. At 2.0 and 3.0 mg/mL, mibefradil caused no detectable inactivation of CYP3A4. At 3.0 mg/mL, none of the MDIs examined caused more than 15% inactivation of cytochrome P450 at inhibitor concentrations that caused extensive P450 inactivation in HLM at 0.1 mg/mL.

Decreased P450 inactivation with the dilution method

To explain why certain MDIs (namely ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil) cause less P450 inactivation when assessed by the dilution method, we postulated that incubating these particular MDIs with a relatively high concentration of HLM is associated with a decrease in the free concentration of inhibitor (due to non-specific binding of the inhibitor to microsomes) or due to inhibitor depletion (due to extensive metabolism), or a combination of both. To test this hypothesis, we examined the metabolic stability of each inhibitor with a low concentration of HLM (0.1 mg/mL for CYP2B6, 2C9, 2C19 and 2D6 assays and 0.05 mg/mL for CYP3A4 assays) and at 10 times this concentration (0.5 or 1.0 mg/mL); the

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former are the incubation conditions for the non-dilution assay, the latter are those for the dilution assay. For this experiment we selected concentrations of inhibitor known to cause a substantial degree of P450 inactivation at 0.05 or 0.1 mg/mL (they were the same concentrations used in Fig. 6) and we measured the concentration of inhibitor every minute over a 15-min period. The results are shown in Fig. 7. *S*-Fluoxetine was essentially metabolically stable at both 0.1 and 1.0 mg/mL. In contrast, substantial inhibitor depletion was observed with the five other MDIs, and the extent of inhibitor depletion increased with increasing concentration of HLM. Ticlopidine was the least stable. At 1.0 mg/mL, ticlopidine was completely metabolized after 2 min and complete metabolism was also observed after a 15-min incubation with 0.1 mg/mL.

For the study depicted in Fig. 7, metabolic stability was evaluated over a 15-min incubation period. However, with both the non-dilution and dilution assays, the usual pre-incubation time is 30 min. We also evaluated the metabolic stability of ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil after a 30-min incubation with HLM at 0.1 and 1.0 mg/mL (or 0.05 and 0.5 mg/mL for the CYP3A4 assays), and the results are summarized in Table 5. Once again, *S*-fluoxetine was the most stable compound; only 21% was consumed after 30 min regardless of the concentration of HLM, making it the only MDI in this group to meet the FDA's recommendation that less than 30% of the inhibitor should be consumed during the incubation period (FDA Draft Guidance for Industry, 2006). After a 30-min incubation with HLM at 0.5 or 1.0 mg/mL, ticlopidine, tienilic acid, azamulin and mibefradil were completely metabolized (≥99%) and 88% of paroxetine was metabolized. Less metabolism was observed at 0.05 or 0.1 mg/mL, but even at these relatively low concentrations of HLM extensive metabolism (>30%) was observed for ticlopidine, paroxetine and mibefradil.

Table 5 also shows the results of experiments to assess the degree of non-specific binding of ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil to HLM at 0.1 and 1.0 mg/mL (or 0.05 and 0.5 mg/mL for the CYP3A4 inhibitors). In all cases, binding to

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microsomes increased with increasing concentration of HLM. Tienilic acid bound to microsomes the least (<15%), followed by azamulin (<25%). Ticlopidine, *S*-fluoxetine, paroxetine and mibefradil all bound extensively (78-86%) to HLM at 0.5 or 1.0 mg/mL; substantial binding (30-64%) was also evident at 0.05 or 0.1 mg/mL.

The final column in Table 5 shows the combined effect of non-specific binding and metabolism on the percent loss of inhibitor. When incubated with NADPH-fortified HLM at 0.5 or 1.0 mg/mL for 30 min, metabolism and non-specific binding caused dramatic decreases in the free concentration of ticlopidine (100%), tienilic acid (100%), *S*-fluoxetine (91%), paroxetine (98%), azamulin (100%) and mibefradil (100%). The corresponding values obtained at 0.05 or 0.1 mg/mL were: ticlopidine (~100%), tienilic acid (~28%), *S*-fluoxetine (52%), paroxetine (74%), azamulin (11%) and mibefradil (97%).

When binding to microsomes is the chief cause for the decrease in cytochrome P450 inactivation, it should be possible to reconcile the shifted IC_{50} values obtained by the dilution and non-dilution methods by basing the IC_{50} values on fu_{mic}, the free concentration of inhibitor In contrast, when inhibitor depletion is the chief cause for the decrease in cytochrome P450 inactivation, it should be possible to obtain lower shifted IC_{50} values (and hence an increase in the magnitude of the IC_{50} shift) by decreasing the concentration of HLM. As shown below, both these predictions were borne out experimentally.

Correcting shifted IC_{50} values for microsomal binding

Of the six MDIs that caused less cytochrome P450 inactivation when incubated with a relatively high concentration of HLM (ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil), only one of them, namely *S*-fluoxetine, was metabolically stable (i.e., less than 30% was consumed during a 30-min incubation with NADPH-fortified HLM at either 0.1 or 1.0 mg/mL). However, *S*-fluoxetine bound non-specifically to HLM. As shown in Table 5, approximately 46 and 78% of fluoxetine bound to HLM at 0.1 and 1.0 mg/mL, respectively. These results suggest that the influence of protein concentration on the shifted IC_{50} values for

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S-fluoxetine – and *only* fluoxetine among this particular group of MDIs – should be fully correctable by taking non-specific binding into account. Table 6 shows the shifted IC_{50} values for ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil determined by the non-dilution method (at 0.05 or 0.1 mg/mL) and the dilution method (at 0.5 or 1.0 mg/mL during the pre-incubation step). When the values were based on the nominal concentration of inhibitor, the shifted IC_{50} values determined by the non-dilution method were all lower than those determined by the dilution method; the ratio of the two values ranged from 0.044 to 0.38. When the shifted IC_{50} values were based on the free concentration of inhibitor (fu_{mic}), the ratio for CYP2C19 inhibition by *S*-fluoxetine approached unity (the actual value was 0.94). Based on the nominal concentration of *S*-fluoxetine, the shifted IC_{50} values determined at 0.1 mg/mL and 1.0 mg/mL were 5.3 and 14 μM, respectively, but these changed to 2.9 and 3.1 μM based on the free concentration of fluoxetine (Table 6). We also measured the rate of CYP2C19 inactivation (k_{obs}) over a wide range of *S*-fluoxetine concentrations (3-100 μM) by the dilution method with a 10 and 40-fold dilution (in which case the pre-incubation concentration of HLM was 1.0 and 4.0 mg/mL, respectively) to mimic the experimental design commonly used to measure K_I and k_{inact}. As shown in Fig. 8, when k_{obs} was based on the nominal concentration of *S*-fluoxetine, the apparent rate of inactivation of CYP2C19 was greatly diminished at the higher protein concentration. However, when the data were processed based on the free concentration of *S*-fluoxetine, which was calculated according to both Hallifax and Houston (2006) and Austin et al. (2002), the rate of CYP2C19 inactivation at 4.0 mg/mL was essentially the same as that at 1.0 mg/mL of HLM. Incidentally, the non-specific binding of *S*-fluoxetine to HLM at 1.0 mg/mL was predicted to be 68% by Hallifax and Houston (2006) and 88% by Austin et al. (2002), which bracket our experimentally determined value of 78% (Table 5).

The effects of decreasing protein concentration on P450 inactivation

The five metabolically unstable MDIs, namely ticlopidine, tienilic acid, paroxetine, azamulin and mibefradil, were evaluated in a non-dilution assay for their ability to inactivate

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cytochrome P450 at the usual concentration of HLM (0.1 mg/mL for CYP2B6, 2C9 and 2C19 and 0.05 mg/mL for CYP3A4) and at one-tenth the usual concentration to test the hypothesis that lowering the concentration of HLM increases the degree of P450 inactivation (as evidenced by a lower shifted IC_{50} value) and, hence, increases the magnitude of the IC_{50} shift. As shown in Table 7, decreasing the concentration of HLM by a factor of 10 lowered the shifted IC_{50} values for CYP2B6 inactivation by ticlopidine (from 47 to 11 nM), CYP2C9 inactivation by tienilic acid (from 66 to 13 nM), CYP2D6 inactivation by paroxetine (from 51 to 25 nM) and CYP3A4 inactivation by azamulin (from 38 to 13 nM) and mibefradil (from 27 to 23 nM). The IC_{50} values for direct inhibition did not change appreciably (they remained within a factor of two) when the concentration of HLM was decreased tenfold. Accordingly, lowering the concentration of HLM by a factor of 10 increased the magnitude of the IC_{50} shift for ticlopidine (from 3.8 to 20 fold), tienilic acid (from 15 to 40 fold), paroxetine (from 15 to 25 fold) and azamulin (from 5.5 to 25 fold) (Table 7). It did not increase the magnitude of the IC_{50} shift for mibefradil because lowering the concentration of HLM from 0.05 to 0.005 mg/mL caused a slight decrease in both the IC_{50} for direct inhibition (IC_{50} curve **B**) and the IC_{50} for MDI (IC_{50} curve **C**).

Effect of substrate incubation time on IC_{50} values

It was noted above that the IC_{50} values for both direct inhibition (curve **B**) and MDI (curve **C**) determined by Obach et al. (2007) were slightly but consistently lower than those determined by Perloff et al. (2009) and us. These systematic differences appear to reflect differences in two experimental conditions, namely the concentration of HLM and the incubation time with the P450 marker substrate. The experimental conditions used by each of the three groups when performing the dilution assay are summarized in Table 8, which shows that the values for both IC_{50} curves **B** and **C** determined by Obach et al. (2007) are generally lower than those determined by Perloff et al. (2009) and us, whereas the magnitude of the IC_{50} shift is generally smaller.

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Obach et al. (2007) generally used a lower concentration of microsomal protein and a longer substrate incubation time to measure cytochrome P450 activity than either Perloff et al. (2009) or we did. Because all three groups used a 10-fold dilution (with the exception of a fivefold dilution for the CYP2C19 assay used by Perloff et al.), the fact that Obach et al. (2007) used a lower final concentration of HLM means they also used a lower concentration of HLM during the pre-incubation step in the dilution assay. Based on the results obtained with ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil (Table 7, Fig. 6), the lower concentration of HLM used by Obach et al. (2009) would be expected to produce lower shifted IC_{50} values (IC_{50} curve **C**), as is indeed the case for all MDIs with the single exception of diltiazem (and this exception was only observed when CYP3A4 activity was measured with midazolam, not testosterone) (Table 8). However, a lower concentration of HLM does not explain why the IC_{50} values reported by Obach et al. (2007) for direct inhibition (IC_{50} curve **B**) are consistently lower than those determined by us and Perloff et al. (2009), but this could be caused by the longer substrate incubation time, which would allow for a greater degree of "unintentional" MDI. (Ideally no MDI should occur during the determination of IC_{50} curve **B**, which is a measure of direct inhibition.) To test this possibility, we determined IC_{50} values for direct inhibition (IC_{50} curve **B**) under our standard conditions (detailed in Table 1) and at one-fourth the protein concentration but at four times the substrate incubation time (to keep the overall extent of substrate metabolism the same). As shown in Fig. 9, decreasing the concentration of HLM and increasing the substrate incubation time caused a decrease in IC_{50} curve **B** for all MDIs examined with the exception of diltiazem (and only when CYP3A4 was measured with midazolam). The results in Fig. 9 suggest that the longer incubation time with the P450 marker substrate (during which the inhibitor is present along with HLM and NADPH) allows each MDI additional time to cause "unintentional" cytochrome P450 inactivation during the measurement of IC_{50} curve **B**.

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Can the non-dilution method fail to detect MDI?

When the dilution method is used and when the data for MDI (IC_{50} curve **C**) are processed "incorrectly" based on the final concentration of inhibitor, the magnitude of the IC_{50} shift for all of the MDIs examined in this study was greater than that determined by the non-dilution method (Table 4). Leaving aside the issue of whether the method of data processing is valid or not, this raises the question of whether the IC_{50} shifts determined by the non-dilution method can be too small to identify MDIs. The non-dilution method certainly succeeded in detecting an IC_{50} shift for all the MDIs examined in this study; all of them exceeded our IC_{50} shift cutoff value of 1.5. Nevertheless, we sought a case where the dilution method (with "incorrect" data processing) could detect MDI and where the non-dilution method could not. The case we selected was the MDI of CYP2C19 by ticlopidine, and this choice was based on a recommendation from Scott Obach and Bob Walsky (Pfizer, Inc.), to whom we are most grateful. Ticlopidine was the most metabolically labile of all the MDIs examined in this study; it was completely metabolized after a 30-min incubation even with 0.1 mg/mL of HLM (Table 5, Fig. 7). We have also shown that the non-dilution method can detect the MDI of CYP2B6 by ticlopidine (Tables 4 and 6). However, ticlopidine is also an MDI of CYP2C19, but it inactivates CYP2C19 less efficiently than it does CYP2B6 (Venkatakrishnan and Obach, 2007). The k_{inact}/K_I for P450 inactivation is 530 $min^{-1}\cdot mM^{-1}$ for CYP2B6 but only 23 $min^{-1}\cdot mM^{-1}$ for CYP2C19 (Venkatakrishnan and Obach, 2007).

We examined ticlopidine as an inhibitor of CYP2C19 under four conditions: (1) the non-dilution method at 0.1 mg/mL (the usual protein concentration), (2) the non-dilution method at 0.01 mg/mL (one-tenth the usual protein concentration), (3) the dilution method at 1.0 mg/mL during the pre-incubation step (and a final concentration of 0.1 mg/mL), and (4) the dilution method at 0.1 mg/mL during the pre-incubation (and a final concentration of 0.01 mg/mL). For the dilution method, the data for IC_{50} curve **C** were processed based on both the final concentration of ticlopidine (as is commonly done) and the initial inhibitor concentration. The

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results are shown in Fig. 10. With the non-dilution method at 0.1 mg/mL, ticlopidine inhibited CYP2C19 and caused a 1.8-fold shift in IC_{50} curves (from 1.05 to 0.59 μM), which exceeded our cutoff value of 1.5 (Fig. 10, panel A). With the corresponding dilution method (with a pre- and post-dilution protein concentration of 1.0 and 0.1 mg/mL, respectively), the shift was 1.7 fold (from 0.57 to 0.34 μM) when the data were processed based on the final inhibitor concentration (Fig. 10, panel C). It is noteworthy that, in theory, the IC_{50} shift for the dilution method should have been 18 fold; i.e., ten times the value obtained by the non-dilution method because of the "incorrect" data processing. With the non-dilution method at 0.01 mg/mL (one-tenth the usual protein concentration), ticlopidine caused a 6.7-fold shift in IC_{50} values (from 0.57 to 0.085 μM) (Fig. 10, panel B). With the corresponding dilution method (with a pre- and post-dilution protein concentration of 0.1 and 0.01 mg/mL, respectively), the shift was 10.7-fold (from 0.78 to 0.073 μM) when the data were processed based on the final inhibitor concentration (Fig. 10, panel D). Once again, in theory, the IC_{50} shift for the dilution method should have been 67 fold. Regardless of whether the initial (pre-incubation) protein concentration was 1.0 or 0.1 mg/mL, when the shifted IC_{50} values from the dilution experiments were processed based on the initial inhibitor concentration, IC_{50} curve **C** was shifted to the *right* of IC_{50} curve **B** (Fig. 10, panel E) or essentially not shifted (Fig. 10, panel F).

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DISCUSSION

The potential for drug candidates to function as MDIs of cytochrome P450 is usually assessed in vitro by examining whether a 30-min incubation of the drug candidate with HLM in the presence of NADPH increases its inhibitory potency (i*.e.,* lowers the value of IC_{50} curve **C**) relative to a 30-min incubation in the absence of NADPH (IC_{50} curve **B**) or a zero-time pre-incubation (IC_{50} curve **A**). A recent PhRMA consensus paper (Grimm et al., 2009) reported that about half the researchers surveyed incorporate into this experimental design a dilution step whereby the samples, after being pre-incubated with a relatively high concentration of HLM (usually 10-fold higher) for 30 min, are diluted (e.g., 10 fold) prior to measuring cytochrome P450 activity. The rationale for the dilution step is that it improves the detection of MDI by increasing the magnitude of the IC_{50} shift ostensibly by lessening the direct inhibitory effect of the drug candidate (Silverman, 1995; Atkinson et al., 2005; Obach et al., 2007; Polasek and Miners, 2007; Fowler and Zhang, 2008; Grime et al., 2009; Grimm et al., 2009; Perloff et al., 2009). In the present study, we examined several known MDIs of cytochrome P450 and determined IC_{50} values **B** and **C** (representing direct inhibition and MDI, respectively) by the non-dilution and dilution method. The results of this study can be summarized as follows:

1. When the dilution method is used, the IC_{50} values for direct inhibition (IC_{50} curves **A** and **B**) vary with the dilution factor unless they are based on the **final** (post-dilution) concentration of inhibitor, as shown in Table 3 and Fig. 2 for fluconazole. This finding can be rationalized on the basis that direct inhibition occurs only in the presence of the P450 marker substrate, which occurs *after* the dilution step (Fig. 1). If the IC_{50} values are based on the initial concentration of inhibitor they increase in proportion to the dilution factor (Table 3 and Fig. 2).
2. In contrast, the IC_{50} values for MDI determined by the dilution method (IC_{50} curves **C**) vary with the dilution factor unless they are based on the **initial** (pre-dilution)

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concentration of inhibitor, as shown for diltiazem and methimazole (Table 3; Figs. 2 and 3). This finding can be rationalized on the basis that MDI occurs during the pre-incubation of the inhibitor with NADPH-fortified HLM, which occurs *before* the dilution step (Fig. 1). It can be further rationalized on the basis that when the dilution method is used to measure the kinetics of inactivation (K_I and k_{inact}), the data are universally processed based on the initial concentration of inhibitor. When the shifted IC_{50} values are based on the final concentration of inhibitor, as they commonly are, the values decrease in proportion to the dilution factor (Table 3; Figs. 2 and 3).

3. In its commonly used format (with all data processed on the final concentration of inhibitor), the dilution method does not produce higher values for IC_{50} curve **B** (i.e., it does not produce less direct inhibition as is commonly assumed) but produces instead lower values for IC_{50} curve **C,** which produces a proportionally larger IC_{50} shift. The lower shifted IC_{50} values give the impression that incubating MDIs with high concentrations of HLM during the pre-incubation phase of the dilution assay leads to *more* P450 inactivation, but this is erroneous and misleading. It disguises the reality that, in many cases, increasing the concentration of HLM results in considerably *less* P450 inactivation (Table 4, Figs. 4-6) due to non-specific binding of the inhibitor to microsomes and/or inhibitor depletion, as shown for ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil (Table 5, Fig. 7).

4. In some – but not all – cases, the shifted IC_{50} values determined by the dilution method matches those determined by the non-dilution method provided the former is based on the initial concentration of inhibitor. This is true for all the MDIs shown in the left-hand side of Fig 4 (furafylline, gemfibrozil glucuronide, diltiazem, troleandomycin and verapamil). However, the shifted IC_{50} values determined by the dilution method does not match those determined by the non-dilution method even if the former are based on the

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initial inhibitor concentration in those cases when the MDI binds non-specifically to HLM and/or is metabolically labile, which is true for all the MDIs shown in the right-hand side of Fig. 4 (ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil).

5. When an MDI is suitably metabolically stable (<30% loss, based on the FDA's criteria) but binds non-specifically to HLM, the IC_{50} values determined by the dilution method can be matched with those determined by the non-dilution method by (a) multiplying the value by the dilution factor and (b) basing the IC_{50} value determined by both the dilution and non-dilution method on the free concentration of inhibitor (fu_{mic}). This was demonstrated for the MDI of CYP2C19 by *S*-fluoxetine (Table 6, Fig. 8).
6. Increasing the concentration of HLM can result in a significant decrease in the extent of P450 inactivation due to non-specific binding and/or inhibitor depletion. A true increase in the extent of P450 inactivation can be achieved by *decreasing* the concentration of HLM, as shown for ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine and azamulin (Table 7, Fig. 10). A low concentration of HLM used in the non-dilution assay produces a true decrease in shifted IC_{50} values and a true increase in the magnitude of the IC_{50} shift, as shown in Table 7 and Fig. 10.
7. Increasing the incubation time with P450 marker substrate (from 5 to 20 min) can result in a sizeable decrease in the value of IC_{50} curve **B** (and curve **A**) due to unintended MDI of cytochrome P450 during the assessment of direct inhibition, as shown in Fig. 9 (and as previously highlighted by Ghanbari et al., 2006). This can reduce the magnitude of the IC_{50} shift.

Based on the results of this study, we recommend that the identification of MDIs be based on a shift in IC_{50} values determined by the non-dilution method because it offers several advantages over the dilution method: it is technically easier because it involves fewer steps; the method of data processing is unambiguous (there is no pre- and post-dilution inhibitor

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concentration to choose from), and the problems associated with non-specific binding and inhibitor depletion are reduced compared with the dilution method. We further recommend that: (1) the concentration of HLM in the non-dilution assay be relatively low (0.1 mg/mL or less); (2) the pre-incubation time with the drug candidate (potential inhibitor) be relatively long (i.e., 30 min or longer); (3) the incubation time with P450 marker substrate be relatively short (5 min or less), and (4) the concentration of drug candidate range up to 10x the total plasma C_{max} at steady state ($C_{max\ ss}$) and the P450 marker substrate concentration be roughly equal to K_m to permit an appropriate estimate of the IC_{50} value for direct inhibition (IC_{50} curves **A** and **B**).

The results in Table 7 and Fig. 10 establish that the degree of P450 inactivation and, hence, the magnitude of the IC_{50} shift can be increased by decreasing the concentration of HLM from 0.1 mg/mL (the concentration widely used in the non-dilution method) to 0.01 mg/mL. Accordingly, an argument could be made that the non-dilution method should be conducted with HLM at 0.01 mg/mL (or even less). Unfortunately, decreasing the concentration of HLM to 0.01 mg/mL may pose an analytical challenge, especially for low turnover substrates like *S*-mephenytoin (CYP2C19). This analytical challenge should not be solved by increasing the incubation time with the P450 substrate, which should be kept relatively short (5 min or less) to prevent unintended P450 inactivation during the substrate incubation period (Fig. 9).

The results of this study raise several questions about the conduct of MDI studies by the dilution method and they have implications for the development of cutoff criteria for MDI.

The dilution method artificially lowers the shifted IC_{50} value, but is this important? Yes it is because the shifted IC_{50} value (the absolute value for IC_{50} curve **C**) has considerable utility, and this utility is lost if the absolute shifted IC_{50} value varies depending on whether it was determined by the non-dilution method or the dilution method, and further varies depending on the actual dilution factor. Shifted IC_{50} values can be used to estimate k_{inact}/K_I (a measure of the

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efficiency of P450 inactivation) based on the following equation (equation 5) from Maurer et al., (2000):

(5) $IC_{50}^{(t)} = \left(\frac{K_I}{k_{inact} \cdot t}\right)\left(1 + \frac{[S]}{K_m}\right)(0.693)$

where *t* is the incubation time with the MDI and [S]/K_m is the ratio of the concentration of P450 marker substrate relative to K_m (the Michaelis-Menten constant). Maurer et al. (2000) validated this relationship experimentally by examining the kinetics of nitric oxide synthase (NOS) inactivation by two MDIs (L-NMMA and L-NIO) based on a non-dilution method. The relationship was also validated for the MDI of P450 enzymes by Berry and Zhao (2008), although they derived a modified version of the Maurer equation (equation 6) to take into account differences in the concentration of HLM to measure IC_{50} shifts (by a non-dilution assay) and the concentration of HLM to determine k_{inact} and K_I (which incorporated a 10-fold dilution):

(6) $\frac{k_{inact}}{K_I} = \left(\frac{0.693}{IC_{50}^{(t)} \cdot t}\right)\left(1 + \frac{[S]}{K_m}\right)\left(\frac{M_{IC50}}{M_{TDI}}\right)$

where M_{IC50} is the concentration of protein in the assay to determine IC_{50} and M_{TDI} is the concentration of protein in the assay to measure k_{inact} and K_I. The ability to estimate k_{inact} and K_I from shifted IC_{50} values (based on a modified Cheng-Prusoff equation) was also validated by Krippendorff et al. (2009) both theoretically (*in silico*) and experimentally using a non-dilution approach in which the rate of formation of fluorescent metabolites (one for CYP1A2, one for CYP3A4) was measured in situ at 2-min intervals (the “progress curve” method).

Obach et al. (2007) reported a strong empirical correlation between shifted IC_{50} values and k_{inact}/K_I (as would be expected from the equations above). The K_I values were determined by the dilution method, and they were based on the initial concentration of inhibitor (as they should be). The shifted IC_{50} values were also determined by the dilution method, but these were based on the final inhibitor concentration; consequently, the shifted IC_{50} values are approximately one-tenth the values based on the initial inhibitor concentration (and many of

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them are likely to be one-tenth of those determined by the non-dilution method). Consequently, if investigators determine shifted IC_{50} values by the non-dilution method and use the relationship between shifted IC_{50} and k_{inact}/K_I published by Obach et al. (2007), their estimates of k_{inact}/K_I could be off by as much as an order of magnitude, assuming a dilution factor of 10 were used.

The "artificially" low shifted IC_{50} values produced by the dilution method can also be a source of confusion when the efficiency of P450 inactivation is considered in terms of the number of moles of inhibitor required to inactivate each mole of P450 enzyme (i.e., the partition ratio). As an example, the inactivation of CYP2C9 by tienilic acid is presented here. The partition ratio for the inactivation of CYP2C9 by tienilic acid is ~12 (Lopez-Garcia et al., 1994), meaning 13 molecules of tienilic acid are consumed for each molecule of CYP2C9 inactivated. Based on CYP quantitation by mass spectrometry (Kawakami et al., 2011), the specific content of CYP2C9 in HLM is approximately 80 pmol CYP2C9/mg protein, hence, an incubation containing HLM at 0.1 mg/mL contains roughly 8 pmol of CYP2C9 (i.e., 8 nM enzyme). This represents the situation in our typical non-dilution assay (Table 1), and under these conditions the shifted IC_{50} value for CYP2C9 inactivation by tienilic acid was 66 nM (Table 4). Assuming for the sake of simplicity the incubation volume was 1 mL, this means 66 pmol of tienilic acid were required to inactivate half of the 8 pmol of CYP2C9 present in the incubation, which is roughly 17 pmol tienilic acid/pmol CYP2C9. When we used the dilution method, the initial concentration of HLM was 1.0 mg/mL; hence, the initial concentration of CYP2C9 was roughly 80 pmol/mL. Based on the initial concentration of tienilic acid, the shifted IC_{50} value for CYP2C9 inactivation was 570 nM (Table 4); hence, 570 pmol of tienilic acid inactivated half the 80 pmol of CYP2C9 in the incubation, which is roughly 14 pmol tienilic acid/pmol CYP2C9, a value that agrees well with that determined by the non-dilution method (17 pmol tienilic acid/pmol CYP2C9). It also agrees with the experimentally determined partition ratio of ~ 12 reported by (Lopez-Garcia et al., 1994; Johnson, 2008), which is not surprising because tienilic acid is

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completely metabolized by HLM at 1.0 mg/mL. If these same calculations are performed with the shifted IC_{50} value based on the final concentration of tienilic acid, the shifted IC_{50} value decreases by a factor of ten to 57 nM (Table 4). Now it appears that half of the 80 pmol of CYP2C9 (the amount of CYP2C9 present in the pre-incubation stage, which is when the enzyme inactivation takes place) is inactivated by only 57 pmol of tienilic acid (under conditions where tienilic acid is completely metabolized); hence, tienilic acid could be mistaken for having a partition ratio close to zero. In other words, if the shifted IC_{50} value for tienilic acid is based on the final (post-dilution) concentration of inhibitor, the dilution assay gives the erroneous impression that tienilic acid is almost a perfect MDI of CYP2C9; one that inactivates the enzyme during a single catalytic cycle. Determining the "true" shifted IC_{50} value (or the magnitude of the "true" IC_{50} shift) also has implications for developing criteria for MDI cutoff values, which is discussed later.

If increasing the concentration of HLM increases the metabolism of the MDI, how can this result in less P450 inactivation? Increasing the concentration of HLM increases the metabolism of all MDIs and yet, in many cases, this results in less cytochrome P450 inactivation (Figs. 4-6). How can increasing the metabolism of the inhibitor result in *less* MDI? To appreciate the reason for this phenomenon one must consider the influence of partition ratio. For example, consider a hypothetical study with an MDI of CYP3A4 (or any other enzyme) that has a partition ratio of 9, meaning that 10 molecules of inhibitor are consumed for each molecule of CYP3A4 inactivated. The other 9 molecules of metabolite are released from the active site either because they fail to inactive CYP3A4 (they miss their target, so to speak) or because they are non-inhibitory metabolites. (Note: A partition ratio of 9 is a realistic number for MDIs of many cytochrome P450 enzymes [Johnson, 2008].) The hypothetical study is conducted with such a low concentration of HLM that the incubation contains a single molecule of CYP3A4. If 10 molecules of inhibitor were added to the incubation, that single molecule of CYP3A4 would be inactivated (i.e., there would be 100% inactivation), and it may take complete

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metabolism of the inhibitor to achieve this. If the concentration of HLM were increased 10 fold (as would be done in the pre-incubation phase of the dilution method) there would be 10 molecules of CYP3A4. If 10 molecules of inhibitor were added, all of the inhibitor would be metabolized, but only one of the 10 molecules of CYP3A4 would be inactivated (i.e., there would be 10% inactivation). The other 9 molecules of CYP3A4 would be spared simply because the concentration of HLM was increased 10 fold. This illustrates the important principle that when the molar ratio of inhibitor to enzyme falls below the partition ratio, the inhibitor will be completely metabolized before all the enzyme is inactivated. In the hypothetical example, all 10 molecules of CYP3A4 could be inactivated by increasing the concentration of inhibitor 10 fold (so that each molecule of CYP3A4 could metabolize 10 molecules of inhibitor), but there is little point performing the dilution assay if it necessitates a corresponding increase in inhibitor concentration.

Implications for cutoff value criteria. The FDA has specified criteria and cutoff values, such as $[I]/K_i < 0.1$ (where [I] is total drug concentration [plasma C_{max} at steady state] and *K*i is the direct (reversible) inhibition constant) for assessing the potential of direct-acting inhibitors to cause clinically relevant cytochrome P450 inhibition, but the agency has not done so for MDI (referred to as TDI in the FDA draft guidance document). The FDA states that "*any time-dependent and concentration-dependent loss of initial product formation rate indicates mechanism-based inhibition*" and that this finding should be followed up with human in vivo studies (FDA Draft Guidance for Industry, 2006). The PhRMA consensus paper on MDI indicates that most respondents use the magnitude of the IC_{50} shift to assess whether a drug candidate is an MDI of cytochrome P450, but the reported cutoff values range from 1.2 to 10 (Grimm et al., 2009). The lower and upper values likely reflect the cutoff values for respondents using the non-dilution method and dilution method, respectively. It is curious that, if the upper cutoff value for the dilution method (10) were corrected for the most commonly used dilution factor (10) so that it might be applied to the non-dilution method, the corrected cutoff value

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would be unity, which would occur when there was NO shift between IC_{50} curves **C** and **B**. This is the first hint that cutoff values cannot be scaled between the non-dilution method and the dilution method simply by applying the dilution factor.

Using the non-dilution method, our criteria for a positive result for MDI is a 1.5-fold shift between IC_{50} curve **C** and either IC_{50} curve **A** or **B**. If we applied this same cutoff to the dilution method (one that incorporated a 10-fold dilution step), this cutoff value should theoretically be set to 15 (10 times larger to account for the 10-fold "artificial" increase in IC_{50} shift). For each of the 14 MDIs shown in Table 4, the IC_{50} shift with the non-dilution method exceeds 1.5. The MDIs that caused the smallest IC_{50} shifts were verapamil-CYP3A4 (3.5-fold shift) and ticlopidine-CYP2B6 (3.8-fold shift). However when the dilution method was used, and when the data were processed based on the final concentration of inhibitor (which amplifies the shift), three MDIs failed to meet a cutoff of 15, namely ticlopidine-CYP2B6 (5.1-fold shift), azamulin-CYP3A4 (11-fold shift) and mibefradil (7.5-fold shift), and one only just exceed it: tienilic acid-CYP2C9 (16-fold shift). In other words, four MDIs that clearly exceed the cutoff value of 1.5 in the non-dilution assay would be classified as non-MDIs or marginal MDIs with the dilution assay if the cutoff were set to 15. Therefore, the cutoff for the non-dilution method (1.5 fold) cannot be applied to the dilution method simply by adjusting the cutoff value for the magnitude of the dilution. The situation is even worse in the case of the inactivation of CYP2C19 by ticlopidine (Fig. 10). Based on the non-dilution method at 0.1 mg/mL, ticlopidine caused a 1.8-fold shift (which narrowly exceeded the 1.5-fold cutoff). Likewise, ticlopidine caused a 1.7-fold shift in the dilution assay (with a 10 fold dilution) when the data were processed based on the final concentration of ticlopidine. This suggests that, even though the dilution method magnifies the IC_{50} shift for several MDIs by a factor of 10 (for a 10-fold dilution), it would be necessary for the dilution method to have the same cutoff value (1.5) as the non-dilution method in order to classify ticlopidine as an MDI of CYP2C19.

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Cutoff values should theoretically vary according to the fold dilution. For example, if the cutoff for the non-dilution method were 1.5, the cutoff for 10-, 20-, 30- and 40-fold dilution should theoretically be 15, 30, 45 and 60, respectively. The foregoing established that this simple relationship cannot be applied without the dilution method failing to identify certain MDIs (those that bind to microsomes and/or are extensively metabolized). The problem is further complicated when one considers not just the fold dilution but the actual concentration of HLM in the pre-incubation phase. As shown in Table 8, the initial concentration of HLM in the dilution method can be as high as 3 mg/mL and as low as 0.25 mg/mL; a 12-fold difference in absolute protein concentration. The problems with non-specific binding and inhibitor depletion are not the same in these two cases. It is not surprising, therefore, that industry cutoffs for MDI vary widely from a 1.2-shift to a 10-fold shift in IC_{50} curves. Developing acceptance criteria and cutoffs for MDI will be extremely difficult with the dilution method because the magnitude of the IC_{50} shift depends on so many factors, but it seems reasonable to assume that they could be developed with the non-dilution method.

Could the non-dilution assay fail to identify an MDI? When the dilution method is used and the data are processed based on the final concentration of inhibitor, the magnitude of the IC_{50} shift is larger than that produced by the non-dilution method, as shown in Table 4 and Fig. 3. In many cases it is 10 times larger (e.g., all the MDIs shown in the left-hand side of Fig. 4), but in other cases it is only slightly larger (e.g., all the MDIs shown in the right-hand side of Fig. 4). This raises the question: Could the non-dilution method fail to identify an MDI that could otherwise be detected by the dilution method? We have yet to identify an MDI that causes a detectable shift in the dilution assay but not in the non-dilution assay (provided we apply a cutoff value of 1.5 to both assays). In an attempt to identify such a compound, we evaluated the inactivation of CYP2C19 by ticlopidine. As shown in Fig. 10, ticlopidine caused a 1.8-fold shift in the non-dilution assay (at 0.1 mg/mL) and a 1.7-fold shift in the dilution assay (with an initial protein concentration of 1.0 mg/mL). When the non-dilution assay was conducted with a lower

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concentration of HLM (0.01 mg/mL), ticlopidine caused a 6.7-fold shift (Fig. 10). Therefore, in general, it is highly unlikely that an MDI that can be identified in the dilution assay could not be identified in the non-dilution assay because the true degree of P450 inactivation increases with *decreasing* protein concentration.

What about other assays that incorporate a dilution step? A dilution step is used in three types of cytochrome P450 inhibition study:

1. An evaluation of IC_{50} shifts to identify MDIs;
2. A determination of the kinetics of P450 inactivation (the measurement of K_I and k_{inact});
3. An assessment of reversibility of MDI (to determine whether MDI involves irreversible P450 inactivation).

These three studies differ in one important aspect: the first one is performed to identify whether a drug candidate is an MDI whereas the latter two are performed after a drug candidate has been identified as an MDI. One practical outcome of this difference is that cytochrome P450 activity is usually measured with a low substrate concentration ([S] = K_m) in the first study (IC_{50} shifts) but with a high substrate concentration in the other two studies (K_I and k_{inact} measurements and an assessment of reversibility). Interestingly, when the dilution method is used to measure K_I and k_{inact}, the method of data processing is not contentious; K_I is universally based on the initial (pre-dilution) concentration of inhibitor, as it should be. However, all three methods involve an initial incubation of the inhibitor with a relatively high concentration of HLM (and NADPH), and the problems surrounding non-specific binding and inhibitor depletion that complicate the assessment of IC_{50} shifts by the dilution method also come into play when the dilution method is used to measure K_I and k_{inact} and assess MDI reversibility. Several of these issues have been reviewed by Ghanbari et al., (2006).

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Should the dilution method be used when measuring K_I and k_{inact}? The argument for incorporating a dilution step in the measurement of K_I and k_{inact} is that it helps to reduce the direct inhibitory effect of MDIs that also cause potent direct inhibition of cytochrome P450. This implies that a dilution step would be particularly useful for potent direct-acting inhibitors that also function as MDIs. Of the MDIs evaluated in this study, those that can also be classified as potent direct-acting inhibitors (those with an IC_{50} curve **B** ≤ 1 μM) are ticlopidine (0.18 μM), tienilic acid (1 μM), paroxetine (0.73 μM), azamulin (0.2 - 0.9 μM) and mibefradil (0.93 μM), as shown in Table 4. All of these compounds are metabolically labile (Fig. 7) and cause considerably *less* cytochrome P450 inactivation when incubated with a high concentration of HLM (Fig. 6). Therefore, the decision to incorporate a dilution step in the determination of K_I and k_{inact} should not be made simply on the basis that a particular MDI also happens to be a potent direct-acting inhibitor. A dilution step should be incorporated in the measurement of K_I and k_{inact} only after it has been established that the shifted IC_{50} value obtained with the dilution method (at 1.0 mg/mL [or the intended concentration of HLM in the pre-incubation step]) is the same as the shifted IC_{50} value obtained with the non-dilution method (at 0.1 mg/mL or less). Alternatively, an ultracentrifugation experiment like that shown in Fig. 6 could be performed to verify that the degree of cytochrome P450 inactivation does not markedly decline when the concentration of HLM is increased 10 or 25 fold.

The dilution method can also increase the apparent K_I value when the high concentration of HLM causes a significant decrease in the free concentration of inhibitor (due to non-specific binding). As shown for *S*-fluoxetine (Table 6, Fig. 8), the impact of microsomal binding can be corrected by expressing shifted IC_{50} and k_{obs} values (and, hence, K_I values) based on the free concentration of inhibitor (fu_{mic}), which can either be measured experimentally or calculated from physiochemical parameters (Austin et al., 2002; Hallifax and Houston, 2006; Gertz et al., 2008).

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Can a dilution step be used to assess MDI reversibility? MDI of cytochrome P450 can involve the formation of metabolites that are (1) more potent reversible (direct-acting) inhibitors than the parent compound, or (2) irreversible inhibitors that bind covalently to the protein of heme moiety of cytochrome P450 (or bind coordinately to the ferrous heme iron, in which case the inhibition is said to be quasi-irreversible) (Ogilvie et al., 2008). The inhibitory effect of the former can be lessened by diluting or dialyzing the sample, whereas the inhibitory effect of the latter cannot. The dilution method always involves incubating the drug candidate with a relatively high concentration of HLM, and this is also commonly done with the dialysis method to offset protein loss due to non-specific binding of microsomes to the dialysis membrane. When these methods are used, it is often assumed that the degree of P450 inactivation observed at a low concentration of HLM will occur to the same extent at a 10- to 25-fold higher concentration. The results shown in Fig. 6 for ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil show that this is a poor assumption. In all these cases, the extent of cytochrome P450 inactivation progressively declined as the concentration of HLM increased from 0.1 mg/mL to 3.0 mg/mL. Consequently, all of these *irreversible* MDIs can easily be misidentified as *reversible* MDIs when assessed by the dilution method or when the assessment by dialysis involves incubating the inhibitor with a high concentration of HLM.

Could the dilution assay fail to identify an MDI? When the potential for a drug candidate to cause MDI is assessed in vitro based on a leftward shift of IC_{50} curve **C** relative to **B**, the non-dilution and dilution method can both identify MDIs provided the data from the dilution method are all processed based on the final concentration of inhibitor. However, the dilution method is something of a balancing act. On the one hand it gains sensitivity by boosting the IC_{50} shifts by processing the data for IC_{50} curve **C** based on the final inhibitor concentration, but on the other hand it often loses sensitivity because the high concentration of HLM in the pre-incubation assay can result in extensive inhibitor depletion and/or a marked decrease in the free

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concentration of inhibitor. Accordingly, when the dilution method is used, there is a greater need to ensure that incubating the drug candidate with a high concentration of HLM in the presence of NADPH for 30 min does not cause extensive metabolism and/or a marked decrease in free concentration. It is perhaps fortunate that many of the drug candidates that have been evaluated previously by the dilution method were screened for metabolic stability in NADPH-fortified HLM, but this is unlikely to apply to *all* drug candidates that have been evaluated by the dilution method in the past.

We do not know whether any drug candidates assayed by the dilution method have been misclassified as non-MDIs, but this could occur if the cutoff criteria were set to 10, as at least one respondent reported in the PhRMA consensus paper (Grimm et al., 2009). With a cutoff value of 10, ticlopidine would not be identified as an MDI of CYP2B6 and CYP2C19 (IC_{50} shift = 5.1 [0.20 → 0.039 μM] and 1.7 [0.57 → 0.34 μM], respectively), and mibefradil (IC_{50} shift 7.5 [0.46 → 0.061 μM]) would not be identified as an MDI of CYP3A4 (Table 4, Fig. 10). The results obtained with mibefradil are illuminating because mibefradil (Posicor) is the only drug withdrawn from the US market largely on the basis of its ability to cause irreversible inactivation of CYP3A4 (Prueksaritanont et al., 1999). When incubated with HLM at 0.1 mg/mL, mibefradil (0.2 μM) caused ~70% inactivation of CYP3A4 (Fig. 6). At 1.0 mg/mL the extent of inactivation decreased to a mere 10%. At 2.0 and 3.0 mg/mL, mibefradil caused no detectable inactivation of CYP3A4. These results argue strongly against the use of high concentrations of HLM to measure the MDI of cytochrome P450.

Many researchers have established automated procedures to evaluate MDI by the dilution method and they have developed a large database on MDI. Accordingly, many researchers will be understandably reluctant to switch immediately to a non-dilution method. Although we do not advocate the dilution method for identifying MDIs, the answers to the

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following questions are intended to guide those researchers who will continue to use the dilution method.

Should differential data processing be applied to the dilution method? In other words, when the dilution method is used to identify MDI of cytochrome P450, should IC_{50} curves **B** and **C** be based on the final and initial concentration of inhibitor, respectively? Perhaps somewhat surprisingly, the answer to this question is NO. When the dilution method is used to identify MDIs of cytochrome P450, all of the data should be processed based on the final concentration of inhibitor. Differential data processing can be applied only after a drug candidate has been identified as an MDI; it cannot be incorporated into a procedure to identify MDIs on the basis of an IC_{50} shift.

Should all the data from the dilution assay be processed based on the initial concentration of inhibitor to prevent the artificial lowering of the shifted IC_{50} values? No, this approach is arguably worse than processing all the data based on the final inhibitor concentration because it would only serve to increase the IC_{50} values for direct inhibition, and an artificial increase in these values would undermine the FDA's criteria for assessing the potential of direct-acting inhibitors to cause clinically significant inhibition of cytochrome P450 (which is based on $[I]/K_i < 0.1$, where K_i can be estimated from the IC_{50} value for direct inhibition).

In summary, we have compared the dilution and non-dilution methods for identifying MDIs of cytochrome P450 (based on IC_{50} shifts) and shown that the ability of the dilution method to produce relatively large IC_{50} shifts is a consequence of basing the shifted IC_{50} values (IC_{50} curve **C**) on the final concentration of inhibitor. This artificial lowering of shifted IC_{50} values (and the corresponding augmentation of IC_{50} shifts) disguises the fact that, for many MDIs, the dilution method actually decreases the extent of P450 inactivation due to inhibitor depletion and/or a decrease in the free concentration of inhibitor. When drug candidates are evaluated in vitro for their ability to cause MDI based on a leftward shift in IC_{50} curve **C** relative to IC_{50} curve

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A or **B**, we recommend that such experiments be conducted with a non-dilution approach (with HLM at 0.1 mg/mL or less) with a cutoff value of 1.5 (for IC_{50} **C**/ IC_{50} **A** or **B**). The non-dilution method offers several advantages over the dilution method: it is technically easier; the method of data processing is unambiguous (there is no pre- and post-dilution inhibitor concentration to choose from), and the problems associated with non-specific binding and inhibitor depletion are reduced. Furthermore, the shifted IC_{50} values determined by the non-dilution method are "true" values (i.e., they are not affected by the dilution factor) and they can be used to estimate K_I/k_{inact} (the efficiency of enzyme inactivation). Perhaps more importantly, the magnitude of the IC_{50} shift determined by the non-dilution method is not augmented by a dilution factor or markedly diminished by inhibitor depletion and/or microsomal binding, hence, they can be used to develop in vitro acceptance criteria and cutoff values to guide the selection of those drug candidates that should be evaluated in vivo for their ability to cause MDI of cytochrome P450.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Parkinson, Kazmi, Buckley, Paris and Ogilvie

Conducted experiments: Kazmi and Yerino

Contributed new reagents or analytic tools: Holsapple, Toren, and Otradovec

Performed data analysis: Parkinson, Yerino, Kazmi, Buckley, Paris, and Ogilvie

Wrote or contributed to the writing of the manuscript: Parkinson, Kazmi, Buckley, Paris and Ogilvie

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Buckley DB, Kazmi F, Yerino P, Toren P, Holsapple J, Ogilvie BW, Paris BL and Parkinson A. 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. 9th International ISSX Meeting; 2010 September 4-8; Istanbul, Turkey. International Society for the Study of Xenobiotics.

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Figure legends

Fig. 1: **Illustration of a typical IC_{50} shift experiment incorporating a dilution step.** The flow diagram represents the typical sequence of incubations when performing a dilution step as part of an IC_{50} shift experiment to identify metabolism-dependent inhibitors of cytochrome P450. Initial pre-incubations are performed with a relatively high concentration of human liver microsomes (typically 1 mg/mL) with zero- and 30-min preincubations (+/- NADPH). Samples are then diluted (typically 10 fold) to a lower concentration of HLM (0.1 mg/mL) for incubation with an appropriate CYP marker substrate. When determining IC_{50} shifts by this method, the metabolism-dependent inhibition component (the 30-min *plus* NADPH samples) occurs prior to the dilution step (during the initial preincubation period); accordingly IC_{50} curve **C** should theoretically be based on the initial (pre-dilution) concentration of inhibitor. Conversely, the direct inhibition component (the zero-min [A] and 30-min minus NADPH [B] samples) occurs after the dilution step (during the incubation with the CYP marker substrate); accordingly IC_{50} curves **A** and **B** should theoretically be based on the final (post-dilution) concentration of inhibitor.

Fig. 2: **The effect of data processing on IC_{50} values determined by the non-dilution and dilution method for fluconazole, a direct-acting inhibitor of CYP3A4, as well as methimazole and diltiazem, metabolism-dependent inhibitors of CYP3A4.** Fluconazole (left), methimazole (middle) and diltiazem (right) were evaluated as CYP3A4 inhibitors based on a non-dilution method (top graphs) and a dilution method that incorporated a 10-, 20-, 30- or 40-fold dilution, as described in *Materials and Methods*. When the dilution method was used, the IC_{50} values were calculated based either on the initial (pre-dilution) concentration of inhibitor (center graphs) or the final (post-dilution) concentration of inhibitor (bottom graphs). The IC_{50} values are listed in Table 3.

Fig. 3: **Inter-laboratory comparison of IC_{50} values determined by the dilution and non-dilution method for diltiazem, a metabolism-dependent inhibitor of CYP3A4.** Diltiazem was evaluated as a metabolism-dependent inhibitor of CYP3A4 (measured with midazolam and testosterone) by a non-dilution method (XenoTech: No dilution) and by a dilution method that incorporated a 10-fold dilution step (XenoTech: 10-fold dilution), as described in *Materials and Methods*. When the dilution method was used, the IC_{50} values were calculated based either on the final (post-dilution) concentration of inhibitor (panel B) or the initial (pre-dilution)

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concentration of inhibitor (panel C). The magnitude of the IC_{50} shift was based on the ratio of the IC_{50} values for direct inhibition (panel A) and the IC_{50} value for MDI based on the final inhibitor concentration (panel A/B) or initial inhibitor concentration (panel A/C). The corresponding data from Perloff et al. (2009) and Obach et al. (2007), both of whom used a 10-fold dilution method, are shown for comparative purposes. The IC_{50} values for all three research groups are shown in Table 4.

Fig. 4: **A comparison of the shifted IC_{50} curves (IC_{50} curves C) determined by the dilution and non-dilution methods for several metabolism-dependent inhibitors of cytochrome P450.** Eleven (11) known metabolism-dependent inhibitors were evaluated as MDI of various P450 enzymes by the non-dilution method (gray circles) and by a dilution method that incorporated a 10-fold dilution step (black circles), as described in *Materials and Methods*. The shifted IC_{50} values (IC_{50} curve C) for the dilution assay are based on the initial (pre-dilution) concentration of inhibitor. Shifted IC_{50} values for both the non-dilution and dilution assays are shown in Table 4. Cytochrome P450 marker substrates are listed in Table 1.

Fig. 5: **The ratio of the shifted IC_{50} value (IC_{50} curve C) determined by the non-dilution method and the dilution method for multiple metabolism-dependent inhibitors.** IC_{50} ratios were calculated as the shifted IC_{50} value without a dilution step divided by the shifted IC_{50} value with a 10-fold dilution step, which was based on the initial (pre-dilution) concentration of inhibitor. The corresponding IC_{50} curves are shown in Fig. 4 and the individual IC_{50} values are shown in Table 4.

Fig. 6: **The effect of microsomal protein concentration on the degree of P450 inactivation by various metabolism-dependent inhibitors.** Ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin and mibefradil were incubated (at the concentrations shown in the figure) with various concentrations of NADPH-fortified pooled HLM (0.1, 0.25, 0.5, 1, 2 and 3 mg/mL) at 37°C for 30 min. The extent of irreversible P450 inhibition was assessed following re-isolation of the microsomes by ultracentrifugation, as described in *Materials and Methods*, followed by measurement of residual P450 activity at 0.1 mg/mL HLM, as described in Table 1.

Fig. 7: **An evaluation of the metabolic stability of selected metabolism-dependent inhibitors at a low and high concentration of human liver microsomes in the presence of NADPH.** The metabolic stability of ticlopidine, tienilic acid, *S*-fluoxetine, paroxetine, azamulin

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and mibefradil (at the concentrations shown in the figure) was evaluated in human liver microsomes at a relatively low protein concentration (0.05 or 0.1 mg/mL; black squares) and high protein concentration (0.5 or 1.0 mg/mL; open circles), as described in *Materials and Methods*. Disappearance of each inhibitor was monitored at 1-min intervals for 15 min by LC/MS/MS as summarized in Table 2.

Fig. 8: **The kinetics of CYP2C19 inactivation (k_{obs}) by *S*-fluoxetine based on the nominal concentration (left) and free concentration of inhibitor (center and right) each with a 10- and 40-fold dilution step.** *S*-Fluoxetine (3 – 100 µM) was preincubated at 37°C for 3, 6, 9, 15 and 30 min with NADPH-fortified human liver microsomes (at 1.0 and 4.0 mg/mL) as described in *Materials and Methods*, after which the samples were diluted 10 or 40 fold (to 0.1 mg/mL) prior to measuring CYP2C19 activity (Table 1). These conditions represent the experimental design commonly used to measure K_I and k_{inact}. The left graph represents the direct plot of the initial rates of inactivation of CYP2C19 (k_{obs}) based on the nominal total concentration of *S*-fluoxetine. The middle and right graphs show the impact of correcting the concentration of *S*-fluoxetine for non-specific binding to microsomes based on the method of Hallifax and Houston (middle graph) and Austin et al. (right graph).

Fig. 9: **Effect of the incubation time with P450 marker substrate in the presence of a metabolism-dependent inhibitor on IC_{50} curve B, a measure of the direct inhibition of cytochrome P450.** IC_{50} values for direct inhibition (IC_{50} curve **B**) were determined for furafylline (0.03 – 30 µM), ticlopidine (0.1 – 100 µM), gemfibrozil glucuronide (0.03 – 30 µM), tienilic acid (0.01 – 5 µM), *S*-fluoxetine (0.03 – 30 µM), paroxetine (0.03 – 30 µM), azamulin (0.1 – 100 µM), diltiazem (0.1 – 100 µM), and verapamil (0.1 – 100 µM) as described in *Materials and Methods*. Condition "X" was based on the concentration of HLM listed in Table 1 with a 5-min substrate incubation period. Condition "Y" was achieved by decreasing the concentration of HLM by a factor of four and increasing the substrate incubation time by a factor four so that the incubation time with marker substrate was increased to 20 min but the overall extent of substrate metabolism remained the same. The values represent the ratio of the IC_{50} values determined under each condition.

Fig. 10: **An evaluation of ticlopidine as a metabolism-dependent inhibitor of CYP2C19 by the non-dilution method (at 0.1 and 0.01 mg/mL) and by the dilution method (at 10x the initial protein concentration).** As described in *Materials and Methods*, IC_{50} values for the

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inhibition of CYP2C19 by ticlopidine were determined under four conditions: (1) the non-dilution method at 0.1 mg/mL (panel A); (2) the non-dilution method at 0.01 mg/mL (panel B); (3) the dilution method at 1.0 mg/mL during the pre-incubation step and a final concentration of 0.1 mg/mL, and (4) the dilution method at 0.1 mg/mL during the pre-incubation and a final concentration of 0.01 mg/mL). For the dilution experiments, the IC_{50} curves for direct inhibition (IC_{50} curve **B**; gray circles) were always based on the final (post-dilution) concentration of inhibitor. The IC_{50} curves for metabolism-dependent inhibition (IC_{50} curve **C**; black circles) were based either on the final (post-dilution) concentration of inhibitor (panels C and D) or the initial (pre-dilution) concentration of inhibitor (panels E and F).

Table 1: Experimental conditions for measuring microsomal cytochrome P450 activity for enzyme inhibition studies

Enzyme	CYP Activity	Substrate concentration (μM)	Protein (mg/mL)	Incubation time (min)	Ionization mode [a]	Mass transition monitored (amu [b])	Internal standard
CYP1A2	Phenacetin *O*-dealkylation	40	0.1	5	ESI+ [c]	152 → 110 [c]	d_4-Acetaminophen
CYP2B6	Bupropion hydroxylation	50	0.1	5	ESI+	256 → 238	d_6-Hydroxybupropion
CYP2C8	Amodiaquine *N*-dealkylation	1.5	0.0125	5	ESI+	328 → 283	d_5-*N*-Desethylamodiaquine
CYP2C9	Diclofenac 4′-hydroxylation	6	0.1	5	ESI–	310 → 266	d_4-4′-Hydroxydiclofenac
CYP2C19	*S*-Mephenytoin 4′-hydroxylation	40	0.1	5	ESI–	233 → 190	d_3-4′-Hydroxymephenytoin
CYP2D6	Dextromethorphan *O*-demethylation	7.5	0.1	5	ESI+	258 → 157	d_3-Dextrorphan
CYP3A4	Testosterone 6β-hydroxylation	50	0.1	5	ESI+ [d]	305 → 269 [d]	d_3-6β-Hydroxytestosterone
CYP3A4	Midazolam 1′-hydroxylation	4	0.05	5	ESI+	342 → 324	d_4-1′-Hydroxymidazolam

[a] Indicates the type of ionization (*i.e.*, electrospray ionization [ESI]) and the polarity (+ or –).
[b] Atomic mass units
[c] Alternative analysis was conducted in ESI– mode with a monitored mass transition of 150 → 107
[d] Alternative analysis was conducted in ESI– mode with a monitored mass transition of 303 → 287

Table 2: Analytical conditions for measuring inhibitor depletion by LC/MS/MS

Inhibitor	Ionization Mode[a]	Mass Transition Monitored (amu)	Internal Standard
Ticlopidine	ESI+	264 → 154	Dextromethorphan
Tienilic acid	ESI -	329 → 274	Probenecid
S-Fluoxetine	ESI+	310 → 44	Probenecid
Paroxetine	ESI+	330 → 192	Dextromethorphan
Azamulin	ESI+	479 → 175	d_6-Dehydronifedipine
Mibefradil	ESI+	496 → 202	d_5-*N*-Desalkylebsatine

Amu, atomic mass units; ESI electrospray ionization

[a] Indicates the type of ionization (*i.e.* ESI) and the polarity (+ or -)

Table 3: Experimentally determined IC_{50} values based on the initial or final inhibitor concentration and the ratio of IC_{50} values determined with dilution (10-40 fold) versus no dilution for fluconazole, a direct-acting inhibitor of CYP3A4, as well as methimazole and diltiazem, metabolism-dependent inhibitors of CYP3A4 based on midazolam 1´-hydroxylation (4 µM)

Dilution Factor	[HLM] (mg/mL) [a]	Fluconazole IC_{50} (µM)				Methimazole IC_{50} (µM)				Diltiazem IC_{50} (µM)			
		30-min Without NADPH		30-min With NADPH		30-min Without NADPH		30-min With NADPH		30-min Without NADPH		30-min With NADPH	
		[Initial]	[Final]	[Initial]	[Final]	[Initial]	[Final]	[Initial]	[Final]	[Initial]	[Final]	[Initial]	[Final]
0	0.05 → 0.05	5.6	5.6	6.6	6.6	570	570	80	80	89	89	18	18
10	0.5 → 0.05	55	5.5	71	7.1	5100	510	76	7.6	780	78	12	1.2
20	1.0 → 0.05	110	5.3	130	6.4	4300	220	71	3.6	1100	57	9.3	0.47
30	1.5 → 0.05	160	5.4	210	6.9	8400	280	64	2.1	1700	58	8.2	0.27
40	2.0 → 0.05	280	6.9	330	8.1	10000	260	59	1.5	2100	52	8.3	0.21

[a] Microsomal protein concentration prior to and after dilution

Note: [Initial] refers to processing the IC_{50} values based on the initial or pre-dilution concentrations of inhibitor, whereas [Final] refers to processing with the IC_{50} values based on the final or post-dilution concentrations.

All values were rounded to two significant figures.

Table 4: Experimentally determined IC_{50} values for various metabolism-dependent inhibitors of human liver microsomal P450 enzymes with and without a 10-fold dilution based on the initial and final concentrations of inhibitor

P450 Enzyme	Substrate	Inhibitor	IC_{50} (μM)									
			No Dilution		With 10-Fold Dilution			Perloff et al., 2009 [c]			Obach et al., 2007 [c]	
			Without NADPH	With NADPH	Without NADPH	With NADPH		Without NADPH	With NADPH		Without NADPH	With NADPH
					[Final]	[Initial]	[Final]	[Final]	[Initial] [b]	[Final]	[Final]	[Initial] [b] [Final]
CYP1A2	Phenacetin	Furafylline	9.6	0.28	3.4	0.36	0.036	8.4	0.21	0.021	1.5	0.27 0.027
CYP2B6	Bupropion	Ticlopidine	0.18	0.047	0.20	0.39	0.039	0.84	0.48	0.048	0.13	0.31 0.031
CYP2C8	Amodiaquine	Gemfibrozil glucuronide	32	2.1	27	2.8	0.28	26	4.6	0.46	ND	ND ND
CYP2C9	Diclofenac	Tienilic acid	1.0	0.066	0.92	0.57	0.057	1.7	0.49	0.049	0.43	0.27 0.027
CYP2C19 [a]	S-Mephenytoin	*S*-Fluoxetine	93	5.3	61	14	1.4	85	15.5	3.1	ND	ND ND
CYP2D6	Dextromethorphan	Paroxetine	0.73	0.05	1.5	0.53	0.053	1.1	0.66	0.066	0.23	0.12 0.012
CYP3A4	Midazolam	Azamulin	0.21	0.038	0.34	0.31	0.031	0.15	0.025	0.0025	ND	ND ND
CYP3A4	Testosterone	Azamulin	0.90	0.095	0.46	0.44	0.044	0.098	0.077	0.0077	ND	ND ND
CYP3A4	Midazolam	Diltiazem	89	18	78	12	1.2	100	34	3.4	54	37 3.7
CYP3A4	Testosterone	Diltiazem	170	32	138	22	2.2	130	28	2.8	55	18 1.8
CYP3A4	Midazolam	Erythromycin	73	13	84	25	2.5	ND	ND	ND	18	12 1.2
CYP3A4	Midazolam	Methimazole	570	80	510	76	7.6	ND	ND	ND	ND	ND ND
CYP3A4	Midazolam	Mibefradil	0.93	0.027	0.46	0.61	0.061	ND	ND	ND	ND	ND ND
CYP3A4	Midazolam	Mifepristone	15	0.53	6.9	0.69	0.069	ND	ND	ND	ND	ND ND
CYP3A4	Midazolam	Troleandomycin	23	0.85	12.3	0.63	0.063	ND	ND	ND	ND	ND ND
CYP3A4	Midazolam	Verapamil	25	7.1	25	4.1	0.41	25	3.4	0.34	12	1.2 0.12
CYP3A4	Testosterone	Verapamil	34	3.0	29	3.8	0.38	28	2.7	0.27	8.2	1.5 0.15

[a] In the case of CYP2C19 a dilution factor of 5-fold was used by Perloff et al., 2009

[b] Calculated based on the reported fold-dilution.

[c] Values published by.

Note: [Initial] refers to processing the IC_{50} values based on an initial or pre-dilution concentration of inhibitor, whereas [Final] refers to processing the IC_{50} values based on a final or post-dilution concentration.

ND no data

Table 5: The effects of microsomal protein concentration on the unbound (free) concentration of selected metabolism-dependent inhibitors and the extent of inhibitor depletion

			Percent Inhibitor Loss		
Inhibitor	[HLM] (mg/mL)	NADPH	HLM Binding [a]	Metabolism After 30 min [b]	Metabolism + HLM Binding [c]
Ticlopidine (0.2 μM)	0.1	—	30	—	30
	1.0	—	81	—	81
	0.1	+	—	98	100
	1.0	+	—	100	100
Tienilic acid (0.5 μM)	0.1	—	0	—	0
	1.0	—	12	—	12
	0.1	+	—	28	28
	1.0	+	—	100	100
S-Fluoxetine (10 μM)	0.1	—	46	—	46
	1.0	—	78	—	78
	0.1	+	—	21	52
	1.0	+	—	21	91
Paroxetine (0.2 μM)	0.1	—	55	—	55
	1.0	—	86	—	86
	0.1	+	—	53	74
	1.0	+	—	88	98
Azamulin (0.1 μM)	0.05	—	2.5	—	2.5
	0.5	—	24	—	24
	0.05	+	—	11	11
	0.5	+	—	99	100
Mibefradil (0.2 μM)	0.05	—	64	—	64
	0.5	—	83	—	83
	0.05	+	—	85	97
	0.5	+	—	100	100

[a] HLM binding was attributed to the observed loss of inhibitor in the absence of NADPH in the post-centrifugation supernatant fraction.

[b] The observed loss of inhibitor due to metabolism alone was determined following a 30 minute incubation with HLM in the presence of NADPH followed by quenching the entire reaction with acetonitrile and assessment of remaining inhibitor.

[c] Contribution of metabolism and HLM binding to depletion of inhibitor. Total inhibitor loss determined following a 30 minute incubation with HLM in the presence of NADPH followed by centrifugation and analysis of remaining inhibitor present in the supernatant fraction.

All values were rounded to two significant figures.

Table 6: Shifted IC_{50} values and IC_{50} ratios corrected for observed microsomal binding

Inhibitor	Enzyme	Total Inhibitor Concentration			Unbound Inhibitor Concentration (fu_{mic})		
		IC_{50} No Dilution [a]	IC_{50} 10-Fold Dilution [b]	IC_{50} Ratio [c]	IC_{50} No Dilution	IC_{50} 10-Fold Dilution	IC_{50} Ratio [c]
Ticlopidine	CYP2B6	0.047	0.39	0.12	0.033	0.076	0.43
Tienilic acid	CYP2C9	0.066	0.57	0.12	0.066	0.50	0.13
S-Fluoxetine	CYP2C19	5.3	14	0.38	2.9	3.1	0.94
Paroxetine	CYP2D6	0.050	0.53	0.094	0.022	0.075	0.29
Azamulin	CYP3A4	0.038	0.31	0.12	0.037	0.23	0.16
Mibefradil	CYP3A4	0.027	0.61	0.044	0.010	0.10	0.10

[a] IC_{50} values from experiments conducted without a dilution step following a pre-incubation of inhibitor with NADPH-fortified HLM. Pre-incubations (30 min) and subsequent marker substrate reactions (5 min) were conducted with 0.1 mg/mL HLM for all enzymes, except CYP3A4 (0.05 mg/mL), with [S] = K_m

[b] IC_{50} values from experiments conducted with a 10-fold dilution step following a pre-incubation of inhibitor with NADPH-fortified HLM. Pre-incubations (30 min) were conducted with 1.0 mg/mL HLM for all enzymes, except CYP3A4 (0.5 mg/mL) Following a 10-fold dilution step, marker substrate reactions (5 min) were conducted with $1/10^{th}$ the concentration of HLM in the pre-incubation with [S] = K_m.

[c] IC_{50} ratio: Shifted IC_{50} no dilution/ Shifted IC_{50} with a 10-fold dilution.

All IC_{50} values are reported as μM, and rounded to two significant figures.

Table 7: The effects of decreasing microsomal protein on the shifted IC_{50} values and the magnitude of the IC_{50} shifts for extensively metabolized metabolism-dependent inhibitors (MDIs)

Inhibitor	CYP Enzyme	[HLM] (mg/mL)	IC_{50} (nM) - NADPH	IC_{50} (nM) +NADPH	IC_{50} Shift [a]
Ticlopidine	CYP2B6	0.1	180	47	3.8
		0.01	220	11	20
Tienilic acid	CYP2C9	0.1	1000	66	15
		0.01	520	13	40
Paroxetine	CYP2D6	0.1	730	51	15
		0.01	880	25	35
Azamulin	CYP3A4	0.05	210	38	5.5
		0.005	330	13	25
Mibefradil	CYP3A4	0.05	930	27	34
		0.005	650	23	28

[a] IC_{50} shift: IC_{50} value -NADPH/IC_{50} value + NADPH.
IC_{50} shift experiments were performed by pre-incubating selected inhibitors with HLM at the stated concentrations for 30 min with or without NADPH. Following the pre-incubation step, marker substrate reactions (5 min) were initiated by the addition of marker substrate ([S] = K_m) without the incorporation of a dilution step.
All values were rounded to two significant figures.

Table 8: A comparison of incubation conditions along with IC_{50}values (and the fold shift) with and without NADPH (curves B and C) for the dilution methods

P450 Enzyme	Substrate Inhibitor	This study					Perloff et al., 2009 [a]					Obach et al., 2007 [a]				
		[HLM] (mg/mL) Time	Fold Dilution	IC_{50} (μM) (B)	IC_{50} (μM) (C)	Fold Shift (B/C)	[HLM] (mg/mL) Time	Fold Dilution	IC_{50} (μM) (B)	IC_{50} (μM) (C)	Fold Shift [b] (B/C)	[HLM] (mg/mL) Time	Fold Dilution	IC_{50} (μM) (B)	IC_{50} (μM) (C)	Fold Shift [b] (B/C)
CYP1A2	Phenacetin Furafylline	0.1 5 min	10	3.4	0.036	94	0.2 10 min	10	8.4	0.021	400	0.03 30 min	10	1.5	0.027	56
CYP2B6	Bupropion Ticlopidine	0.1 5 min	10	0.20	0.039	5.1	0.1 5 min	10	0.84	0.048	18	0.05 20 min	10	0.13	0.031	4.2
CYP2C8	Amodiaquine Gemfibrozil glucuronide	0.0125 5 min	10	27	0.28	96	0.02 5 min	10	26	0.46	57	0.025 10 min	10	ND	ND	ND
CYP2C9	Diclofenac Tienilic acid	0.1 5 min	10	0.92	0.057	16	0.05 5 min	10	1.7	0.049	35	0.03 10 min	10	0.43	0.027	16
CYP2C19	S-Mephenytoin *S*-Fluoxetine	0.1 5 min	10	61	1.4	44	0.3 10 min	5	85	3.1	27	0.2 40 min	10	ND	ND	ND
CYP2D6	Dextromethorphan Paroxetine	0.1 5 min	10	1.5	0.053	28	0.1 5 min	10	1.1	0.066	17	0.03 10 min	10	0.23	0.012	19
CYP3A4	Midazolam	0.05 5 min	10	0.34	0.031	11	0.02 5 min	10	0.15	0.0025	60	0.03 4 min	10	ND	ND	ND
	Azamulin															
	Diltiazem			78	1.2	65			100	3.4	29			54	3.7	15
	Verapamil			25	0.41	61			25	0.34	73			12	0.12	100
CYP3A4	Testosterone	0.1 5 min	10	0.46	0.044	10	0.05 10 min	10	0.098	0.0077	13	0.03 10 min	10	ND	ND	ND
	Azamulin															
	Diltiazem			138	2.2	63			130	2.8	46			55	1.8	31
	Verapamil			29	0.38	76			28	0.27	104			8.2	0.15	55

[a] Values published by.
[b] Calculated based on published values.
Note: All IC_{50} values are based on the final or post-dilution concentrations.
ND no data

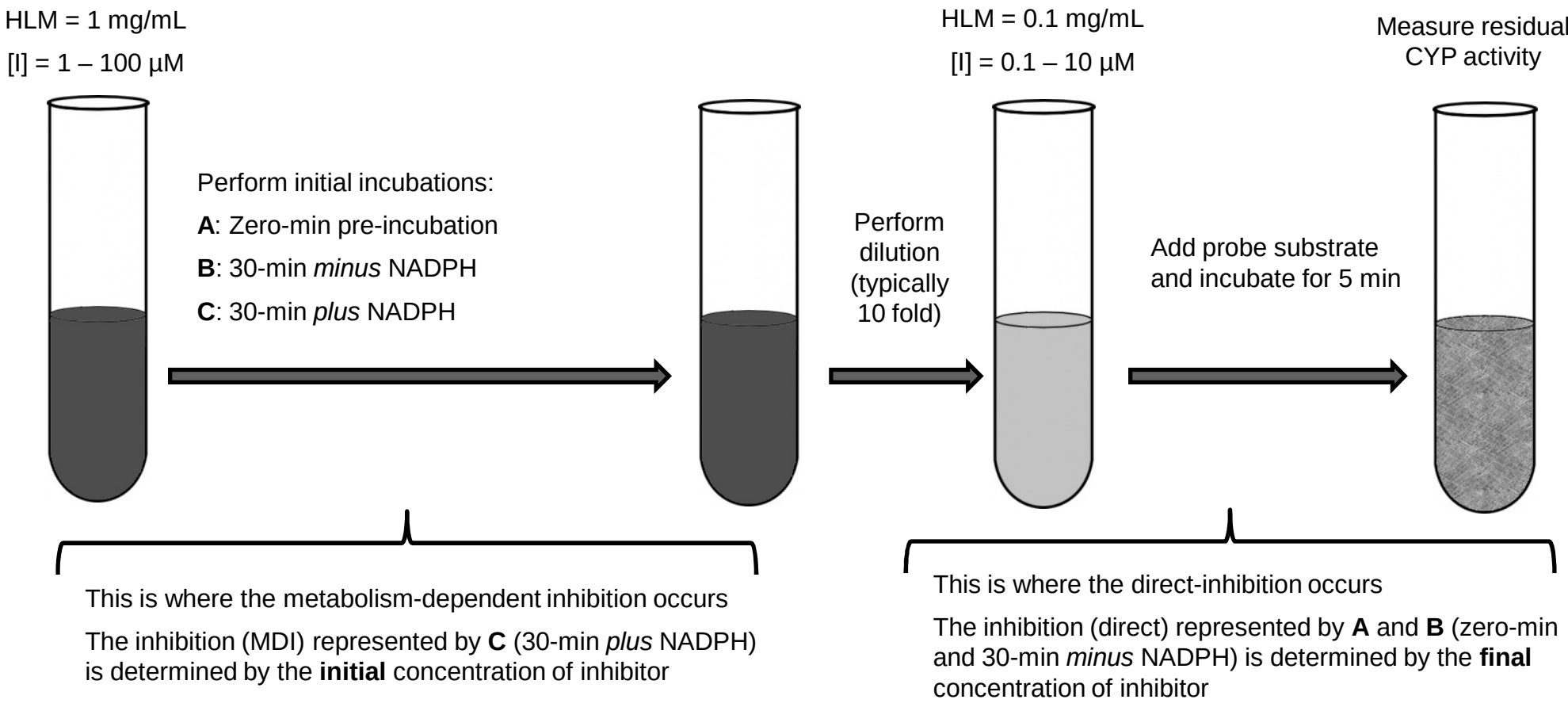


Figure 1

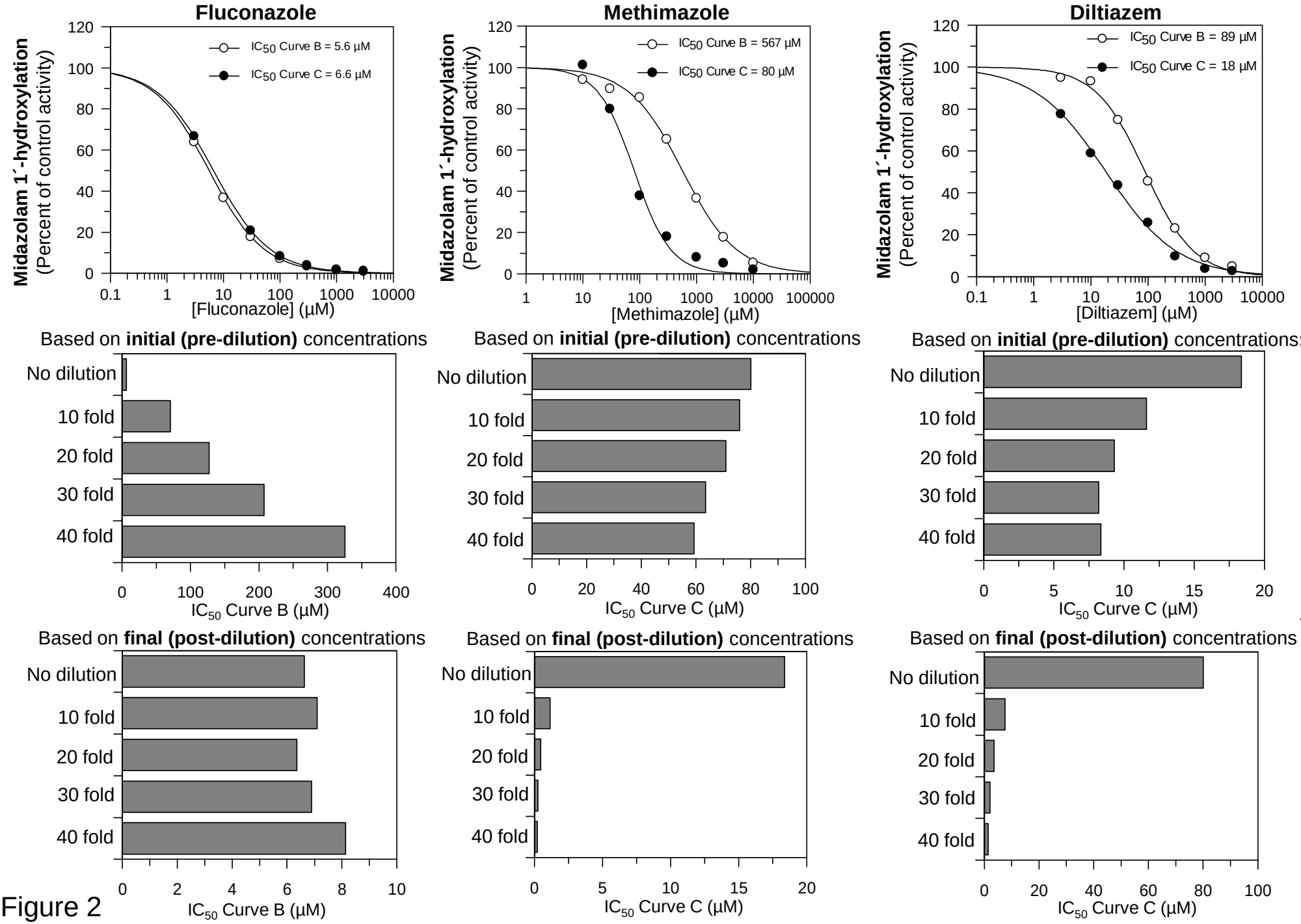


Figure 2

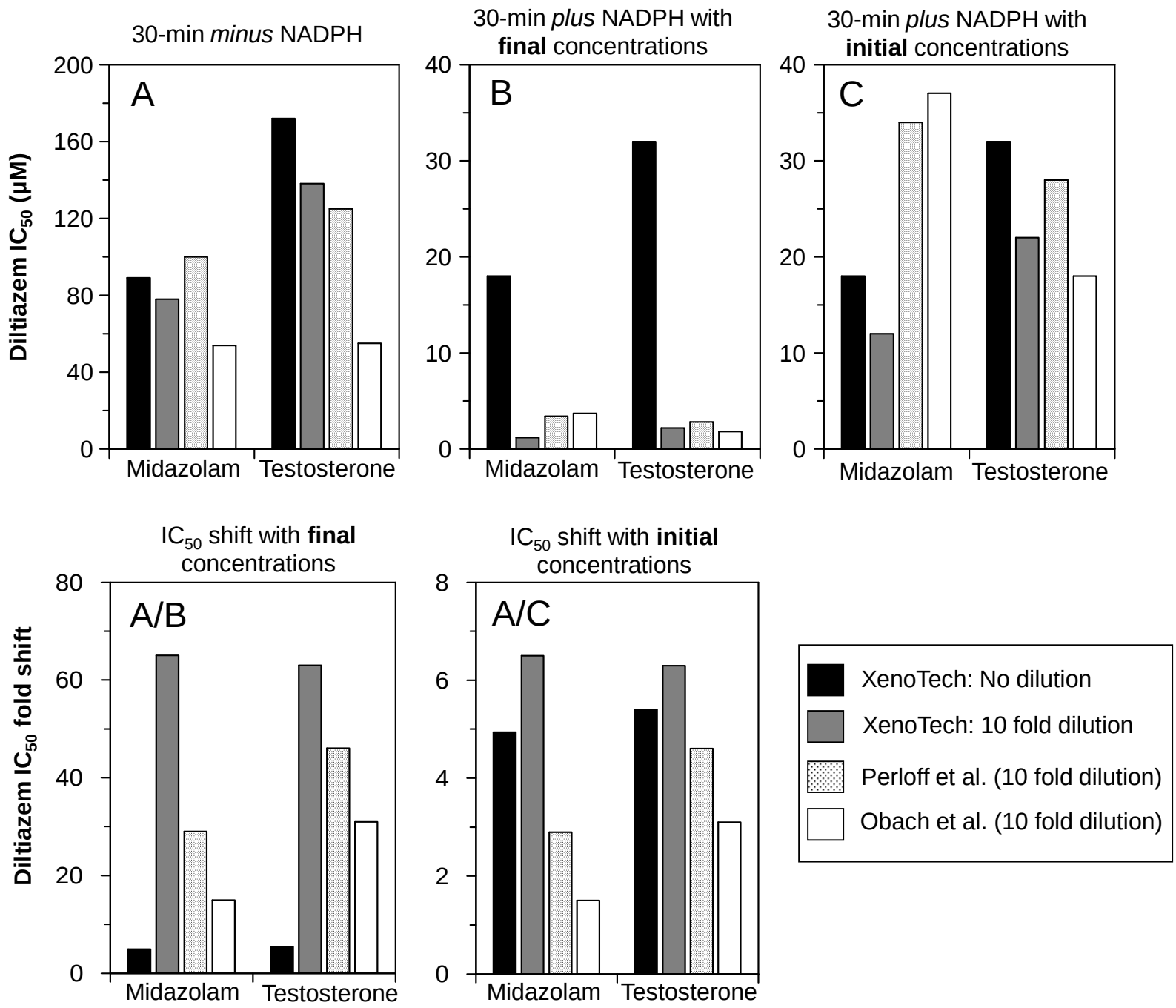


Figure 3

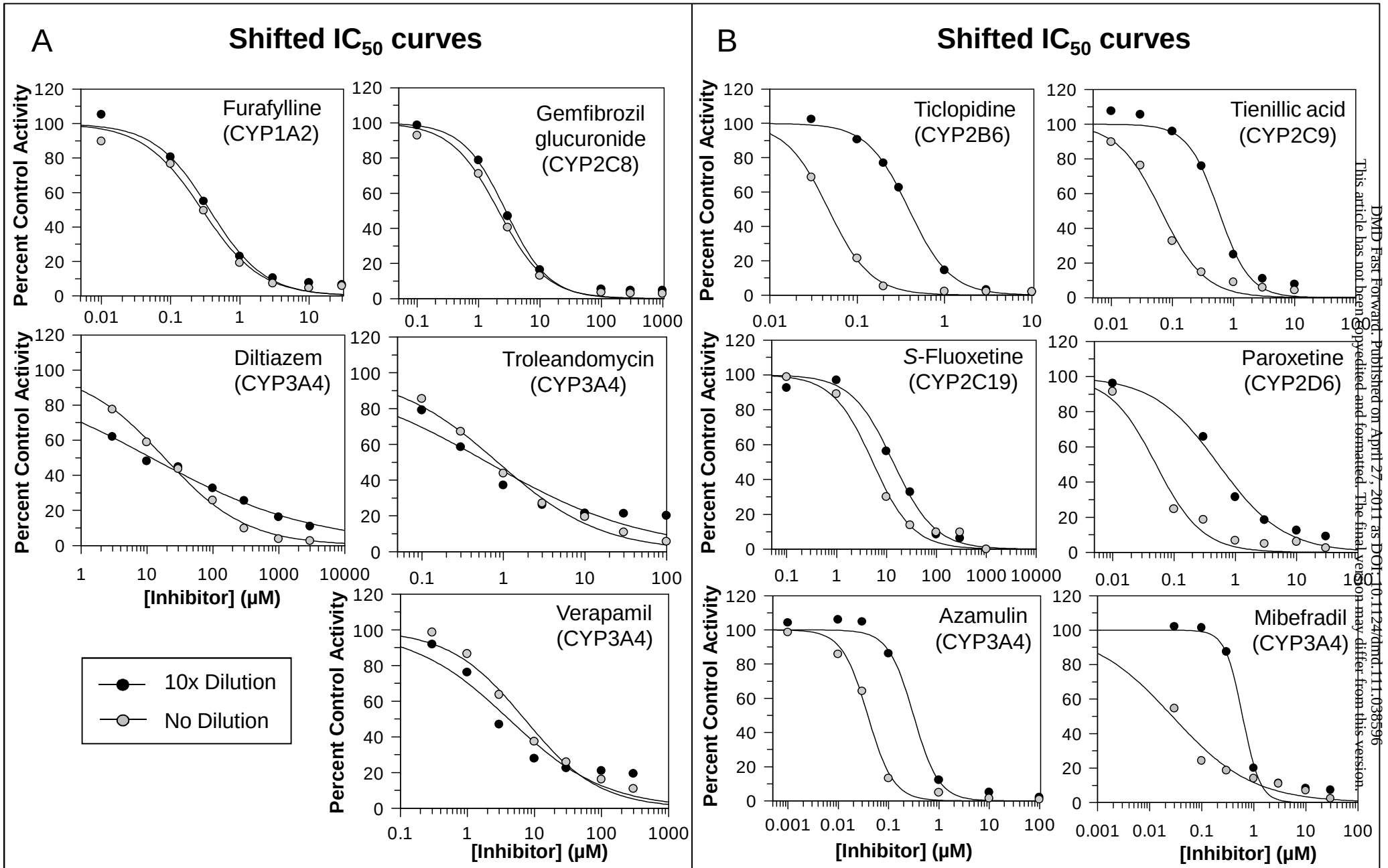


Figure 4

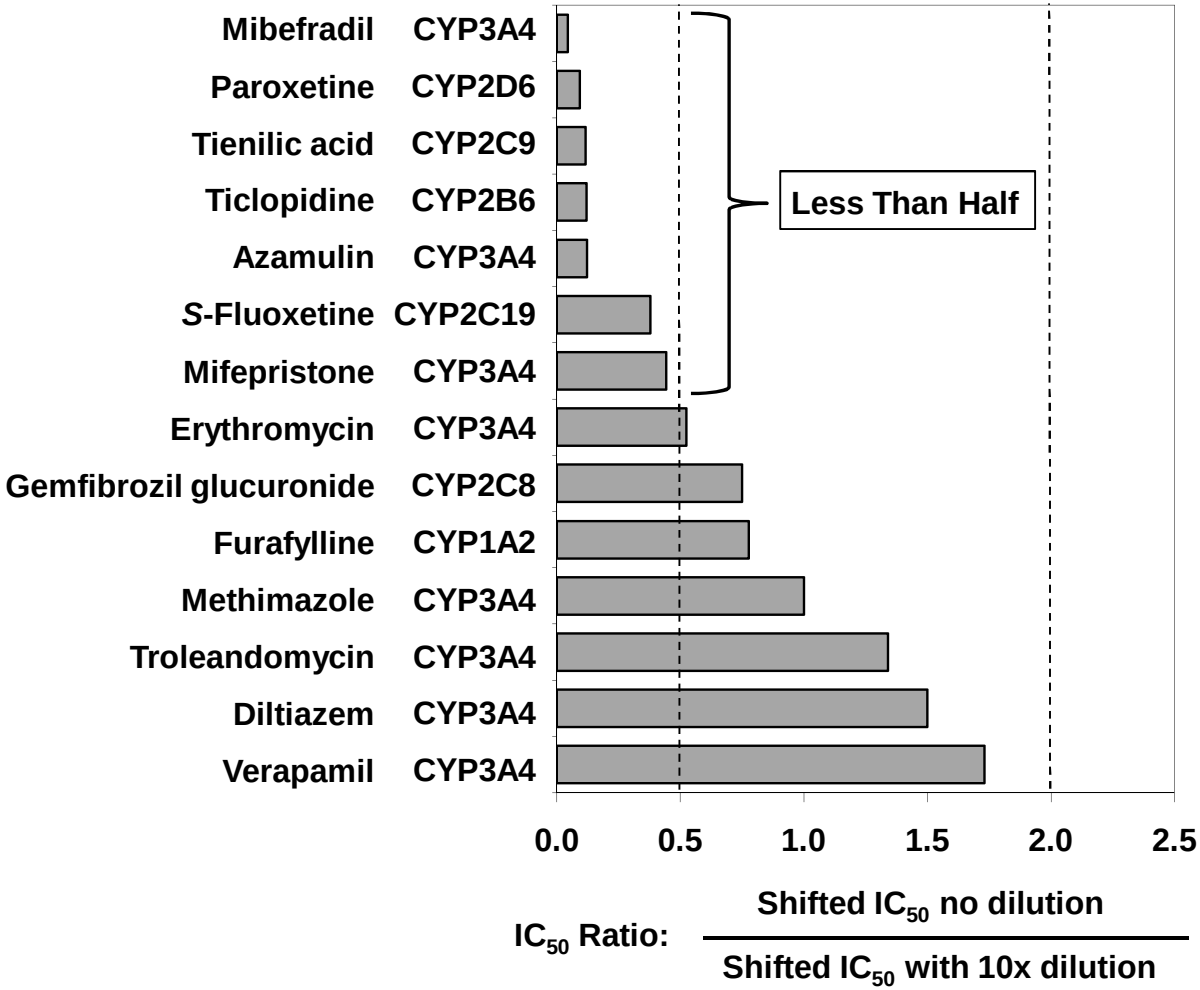


Figure 5

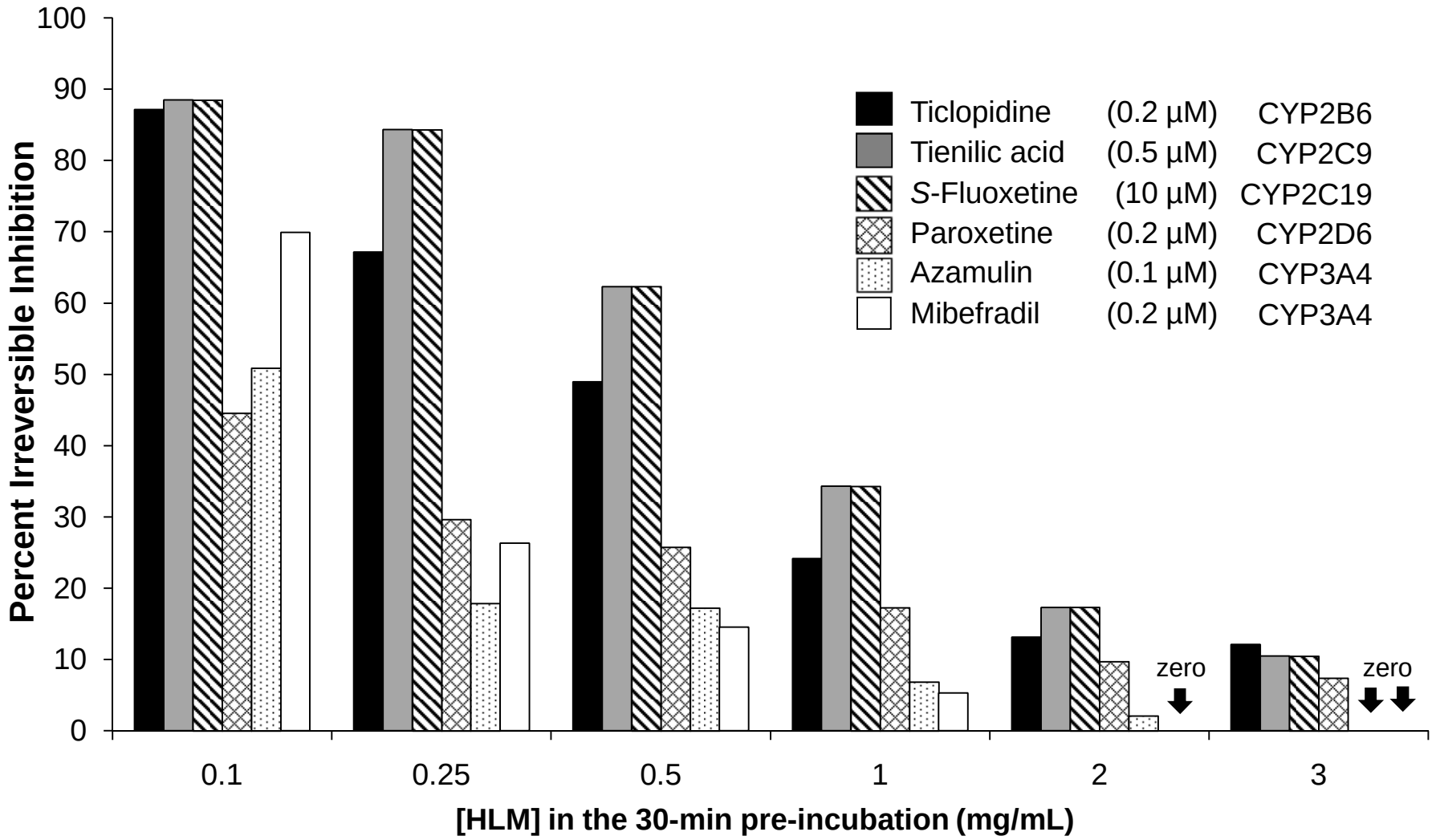


Figure 6

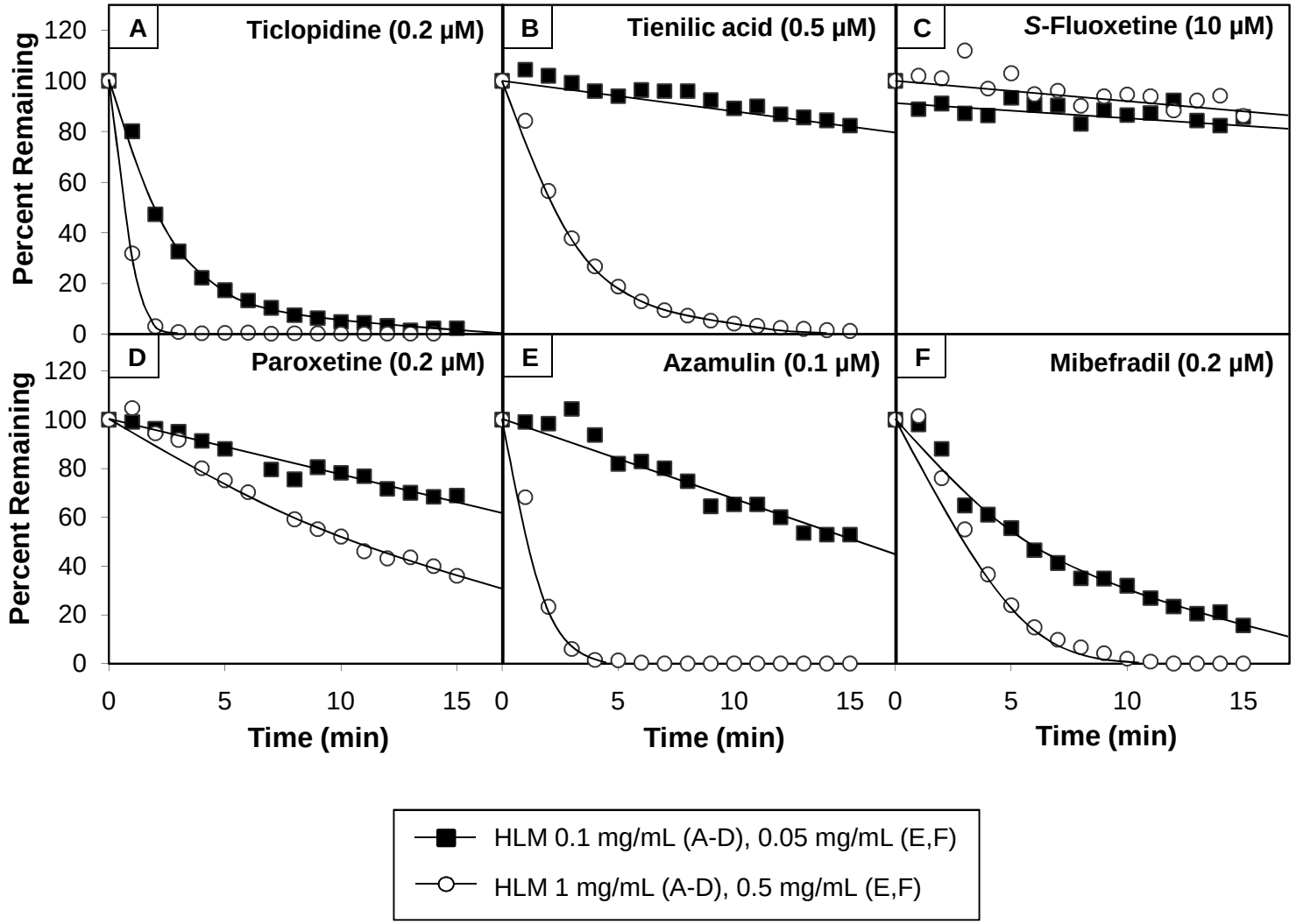
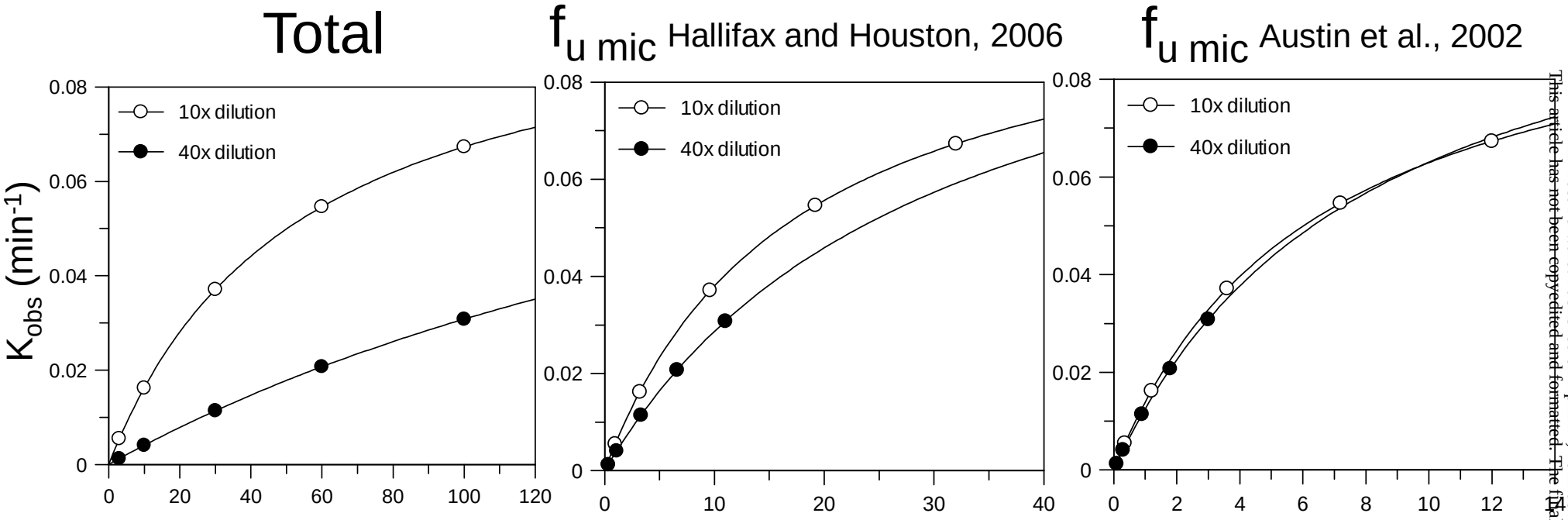


Figure 7



	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (min^{-1} mM^{-1})
10 fold (1.0→0.1)	0.104	53.7	1.9
40 fold (4.0→0.1)	0.115	274	0.4

k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (min^{-1} mM^{-1})
0.104	17.2	6.0
0.115	30.1	3.8

k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (min^{-1} mM^{-1})
0.104	6.5	16
0.115	8.2	14

Figure 8

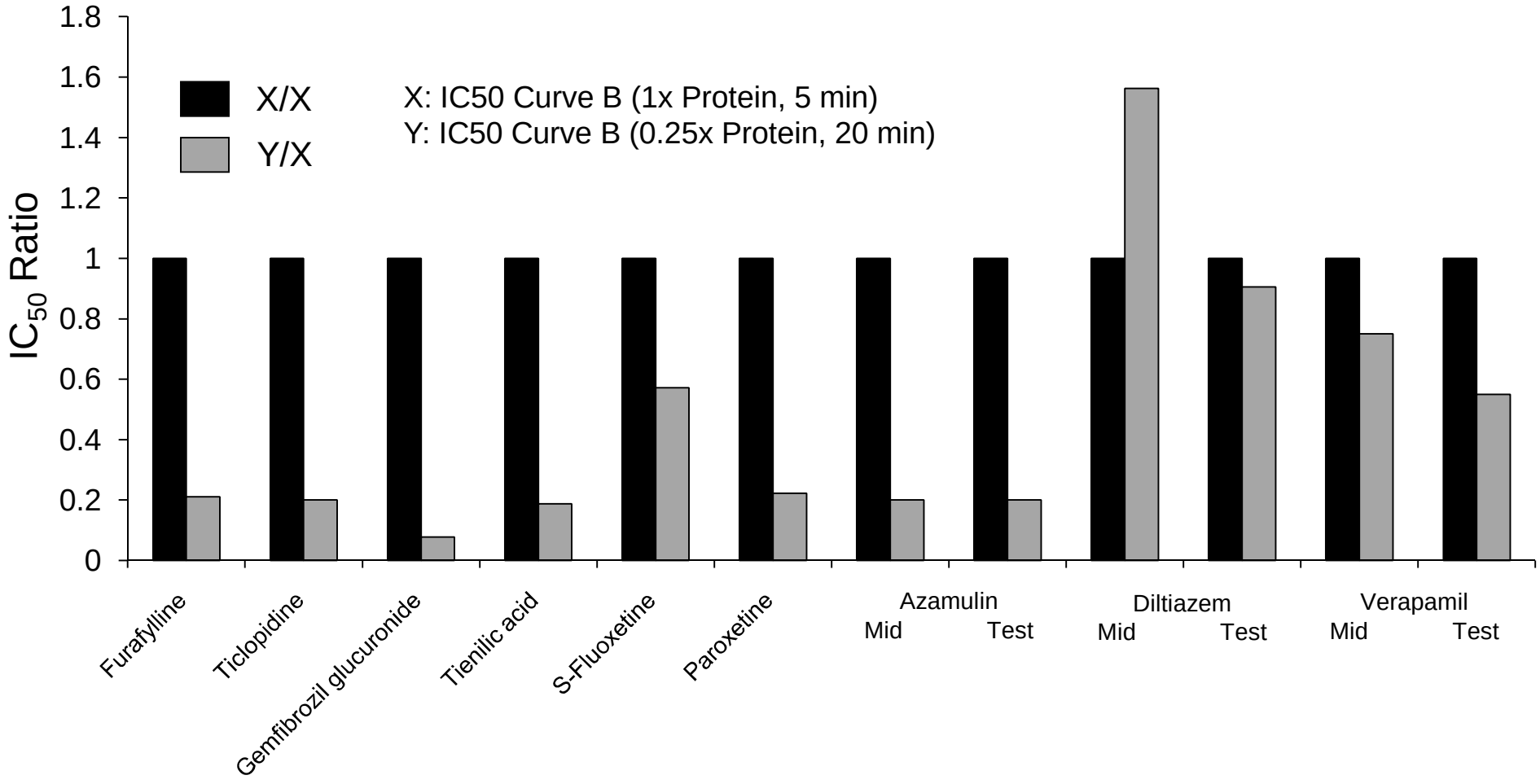


Figure 9

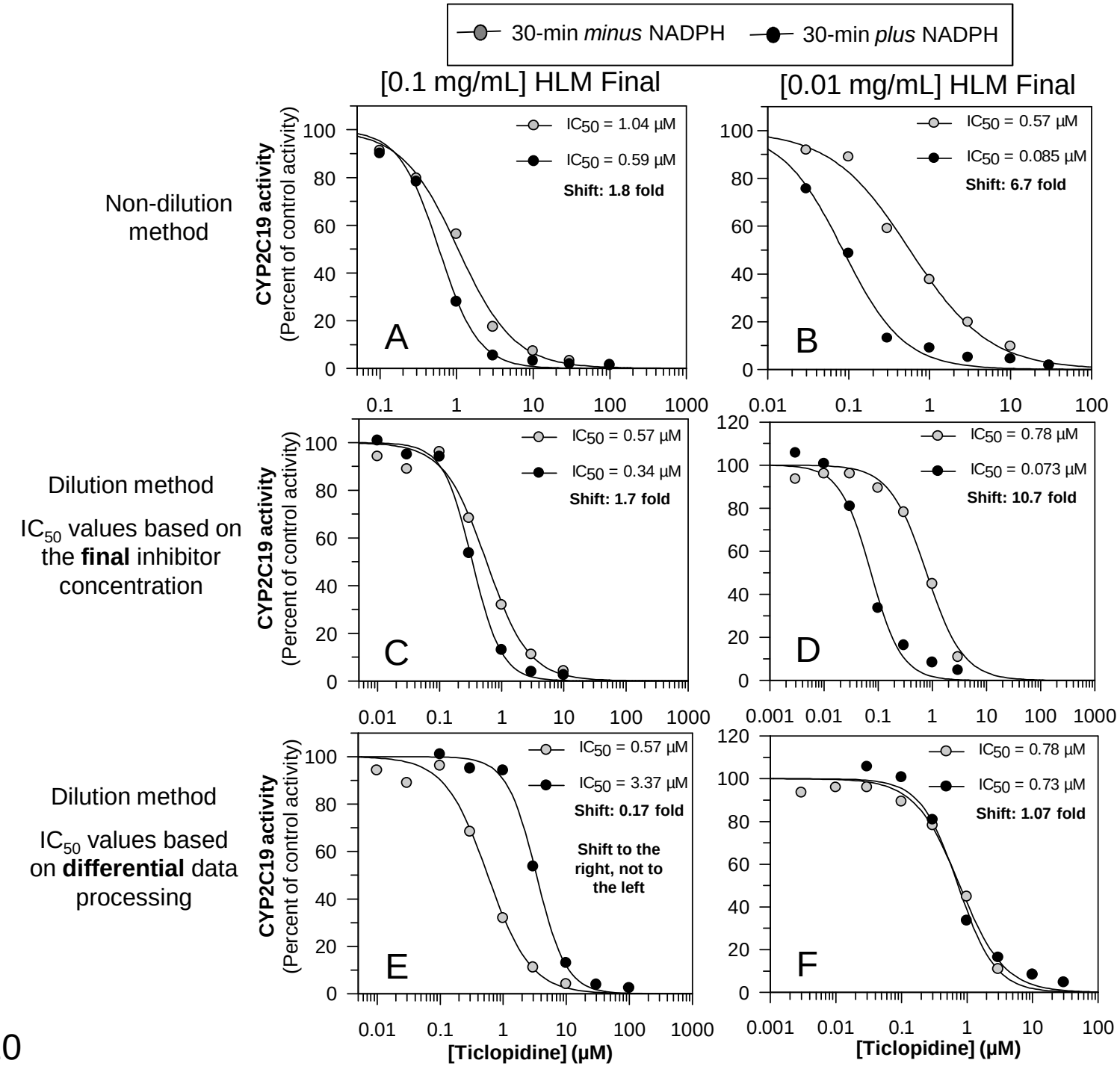


Figure 10