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# Automated Assessment of Time-Dependent Inhibition of Human CYP Enzymes using LC-MS-MS Analysis

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Running title: Automated CYP TDI Assays with an LC-MS-MS Endpoint

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Abbreviations

MDMA, methylenedioxy-n-methylamphetamine

NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form

HLM, human liver microsomes

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**Abstract**

Increasing reports of time-dependent inhibition of cytochrome P450 suggest further emphasis on interpreting the consequences, either from a pharmacokinetic or toxicological perspective. Two automated, time dependent inhibition assays with an LC-MS-MS end-point are presented. The initial assay utilises human liver microsomes, a single concentration of inhibitor and a single pre-incubation time of thirty minutes. Phenacetin, diclofenac, S-mephenytoin, bufuralol and midazolam are used as substrates for CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 and the assay differentiates between reversible and irreversible inhibition. The second assay uses individual recombinant human CYPs, six inhibitor concentrations and three time points to accurately define $k_{inact}$ and $K_I$. A good correlation is demonstrated between $k_{inact}/K_I$ and partition ratio, indicating that both terms are related in describing the efficiency of enzyme inactivation. Despite the single pre-incubation time point of thirty minutes used in the initial assay, a good relationship has been found to exist between the unbound $IC_{50}$ estimated from this initial screen and the $k_{inact}/K_I$ ratio derived from the more extensive subsequent single CYP assay. The higher throughput human liver microsomal assay can therefore generate $IC_{50}$ values which can be used to predict the pharmacokinetic impact on co-therapies from the estimated $k_{inact}/K_I$ ratio, predicted human dose and pharmacokinetics.

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## Introduction

Drug-drug interactions (DDIs) following drug therapy, which result in a high number of hospital admissions and even deaths (Kohler, *et al.*, 2000; Lazarou *et al.*, 1998) are primarily caused by macromolecule binding of reactive species or drug co-therapy resulting in plasma concentrations of one of the co-administered drugs being elevated to toxic levels (Hollenberg, 2002). The mechanism is frequently competition at the active site of drug metabolising enzymes. Since multiple drug therapy is a very common practice, the possibility of DDI therefore exists in the majority of patients. This is a high profile issue for drug discovery and development programs and there is even an on-line DDI database that shows the number of drugs currently implicated (Carlson *et al.*, 2002). Great importance is now placed on *in vitro* studies as tools for predicting *in vivo* DDIs, particularly those resulting from cytochrome P450 (CYP) inhibition (Lin & Lu, 1998), since the metabolic elimination of a large number of drugs is dependent on the CYP family of enzymes.

To date 57 human cytochrome P450 genes have been identified http://drnelson.utmem.edu/CytochromeP450.html), but remarkably only three human CYPs (3A4, 2C9 and 2D6) perform the majority of biotransformations involving pharmaceuticals (Smith *et al.*, 1998). CYP1A2, 2B6, 2C8, 2C19 and 2E1 are also involved, but to a much lesser extent. Inhibition of CYP-dependent metabolism can generally be classified into three categories: reversible, quasi-irreversible (when compounds complex the heam prosthetic group and leave the CYP functionally inactive) and irreversible or mechanism-based (compounds covalently bind to the heam or the surrounding protein) (Hollenberg, 2002). Reversible CYP inhibition screening has been commonplace within drug discovery functions for some time, but reports of time dependent inhibition (TDI) are increasing in prevalence, indicating that more emphasis is now being placed on interpreting the effects, either from a pharmacokinetic or toxicological perspective. Moreover, irreversible and quasi-irreversible inhibition are often viewed as more serious than reversible inhibition, since the inhibitory effect remains after elimination of the parent drug from the body. TDI is an unusual



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occurrence with most enzymes, but it is observed at a higher frequency in CYP catalysed reactions, perhaps due to the reactivity of the oxygenated species formed during the course of the oxygenation reactions (Hollenberg, 2002). There are examples of irreversible or quasi-irreversible CYP inhibition across many classes of therapeutic drugs, recreational drugs and herbal medicines (Zhou *et al.*, 2005) and all of the major drug metabolising CYPs have been implicated (Zhou *et al.*, 2004; Polasek *et al.*, 2004; Lu *et al.*, 2003; Fan *et al.*, 2003; Bertelsen *et al.*, 2003; Ha-Duong *et al.*, 2001; Palamanda *et al.*, 2001; Chun *et al.*, 2001a; Chun *et al.*, 2001b; Koenigs & Trager, 1998; Newton *et al.*, 1995; Lopez-Garcia *et al.*, 1994; Kunze & Trager, 1993)

In a Drug Discovery setting, three different analytical end-points are typically employed for the study of CYP inhibition *in vitro*: liquid scintillation counting of radioactivity liberated during site-specific metabolism (Moody *et al.*, 1999); selective analysis of fluorescent metabolites (Crespi & Stresser, 2000) or mass spectrometry (Weaver *et al.*, 2003; Yin *et al.*, 2000). Whilst all these formats are available in this laboratory, two automated time dependent inhibition assays are presented which employ an LC-MS-MS end-point. The initial assay utilises human liver microsomes, a single concentration of inhibitor and a single incubation time. The assay uses industry recommended CYP substrates (Bjornsson *et al.*, 2003), is shown to differentiate between reversible and irreversible inhibition and to give a good prediction of $k_{inact}/K_I$ (kinetic parameters of time dependent inhibition). The second assay uses single recombinant human CYPs, six inhibitor concentrations and three time points to define $k_{inact}$ and $K_I$ accurately.

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## Materials and Methods

**Chemicals.** Furafylline, ticlopidine, troleandomycin, mifepristone, 6ß-hydroxy testosterone, testosterone, methylenedioxy-n-methylamphetamine (MDMA), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), phenacetin, and diclofenac (sodium salt), were purchased from Sigma-Aldridge (Poole, Dorset, UK). S-Mephenytoin, bufuralol (hydrochloride salt) and midazolam (hydrochloride salt) were purchased from Ultrafine Chemicals (Manchester, UK).

Dimethylsulphoxide (DMSO), formic acid, acetonitrile and methanol (MeOH) were purchased from Fisher Scientific (Loughborough, UK). Tienilic acid was synthesised at AstraZeneca Charnwood.

**Source of Human Liver Microsomes and Cytochrome P450 Enzymes.**

The HLM pool (48 donors) was supplied by BD Gentest (Oxford, UK). *Escherichia coli* membranes co-expressing human CYP and human NADPH-P450 reductase (1A2LR, 2C9R, 2C19R, 2D6R, 3A4LR) were purchased from CYPEX (Dundee, UK).

**Substrate Selectivity.**

With the possible exception of phenacetin (O-deethylation), the CYP probe reactions used in this study are CYP-selective when the substrate concentrations are equivalent to their Km values (Weaver *et al*., 2003). However, in order to minimise the impact of reversible inhibition (since $IC_{50}$ = Ki x (1 + substrate concentration/Km) for competitive inhibition, Segel, 1975), substrate concentrations equivalent to 4x the Km values were employed for the time dependent inhibition assays. Using these elevated concentrations (75 µM, 10 µM, 100 µM, 40 µM and 10 µM for phenacetin, diclofenac, S-mephenytoin, bufuralol and midazolam), the selectivity of each CYP probe reaction was investigated using individual CYPs at enzyme concentrations intended to mimic those present in the human liver microsomal preparation (40, 60, 15, 5 and 100 pmoles/ml for CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 respectively, Soars *et al*., 2003). Incubations were performed in a 37°C water bath in glass vials containing NADPH (1 mM), recombinant human CYP, substrate and phosphate



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buffer (0.1 M, pH 7.4). After 10, 20 and 30 minutes aliquots (100 µl) were removed and pipetted into 1.2 ml polypropylene tubes containing ice cold methanol (100 µl). The samples were centrifuged and the supernatant analysed by LC-MS-MS, as described below.

**Automated Time Dependent Inhibition (TDI) assay using HLM**

The fully automated TDI assay using human liver microsomes is performed using a Genesis robot (Tecan, Reading, UK) running Gemini software. The assay is designed to run eighteen compounds (12 test compounds + 6 standard inhibitors). The Tecan bed layout contains a sample rack, two incubation plates, a reagent rack and a quench rack. The sample rack consists of 1.2 ml polypropylene tubes in an 8 x 12 (96-well format) box (VWR International, Poole, UK). The tubes contain test and standard inhibitor stocks (100x the initial incubation concentration in DMSO). The standard inhibitor stock solution concentrations are as follows: furafylline (1 mM), tienilic acid (0.05 mM), ticlopidine (0.5 mM), MDMA (0.5 mM), troleandomycin (0.15 mM) and mifepristone (0.5 mM). Initial investigations with test compounds use 1mM stocks (10 µM pre-incubation concentration). The two incubation plates are 96-well polypropylene microplates (Corning Life Sciences, Netherlands). The reagent rack consists of two polypropylene inserts, each containing two reagent reservoirs, (Biomek reservoirs, purchased from Beckman, USA) within an aluminium block heated to 37$^{o}$C. The separate reservoirs contain HLM, NADPH and substrate solution. The quench rack consists of 1.2 ml polypropylene tubes in an 8 x 12 (96-well format) box (VWR International, Poole, UK).

The assay runs a pre-incubation (30 minutes, 1 mg/ml HLM incubation containing 1 mM NADPH and test inhibitor) and a secondary incubation (containing an aliquot from the pre-incubation, 1 mM NADPH and substrates for CYPs 1A2, 2C9, 2C19, 2D6 and 3A4). The secondary incubation runs for 15 minutes. The substrates are prepared manually: aliquots of methanolic stocks of phenacetin (50 µL of a 8.96 mg/ml stock), diclofenac (50 µL of a 2.12 mg/ml stock), S-mephenytoin (50 µL of a 14.56 mg/ml stock), bufuralol (50 µL of a 7.94 mg/ml stock) and midazolam (50 µL of a 2.42 mg/ml stock) are pipetted into a 20 ml glass



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scintillation vial, evaporated to dryness under nitrogen gas and stored at –20°C until required. On the day of the assay, the dried substrate cocktail is resuspended in phosphate buffer (20 ml, 0.1 M, pH 7.4) by vortexing and sonication (20 minutes in a sonic bath at 37°C).

The pre-incubation plate comprises HLM (195 µl, 1.3 mg/ml microsomal protein) and aliquots (2.5 µl) of inhibitor stock solution or DMSO for the control incubations. The pre-incubation is initiated by the addition of NADPH (52.5 µL, 5 mM) to the left-hand half of the plate and phosphate buffer (52.5 µL, 0.1 M, pH 7.4) to the right-hand half of the plate. The pre-incubation plate is then moved into a Tecan MIO incubator set at 37°C. During this pre-incubation stage, the secondary incubation plate is prepared: the substrate cocktail (150 µl) is added to all wells and a further aliquot of NADPH (50 µl, 5 mM) is added. At the end of the 30 minute pre-incubation, an aliquot of this incubation (50 µl) is removed and added to the secondary incubation plate. The final secondary incubation concentration of the substrates is as follows: 75 µM phenacetin, 10 µM diclofenac, 100 µM S-mephenytoin, 40 µM bufuralol, 10 µM midazolam. The secondary incubation progresses for 15 minutes in a Tecan MIO incubator set at 37°C prior to quenching: An aliquot (50 µl) is removed from the incubation and pipetted into the tubes in the quench rack (containing 100 µl of methanol). Quenched samples are chilled at –20°C for 2 hours, centrifuged at 2000 g for 15 minutes and the supernatants transferred to microtitre plates for LC-MS-MS analysis.

**Automated TDI Assay using Individually Expressed Human Cytochrome P450 Enzymes to Determine $K_I$ and $k_{inact}$.**

The fully automated $K_I$ and $k_{inact}$ TDI assay is designed to run three compounds (two test compounds plus one standard inhibitor). The incubation is performed in phosphate buffer (0.1 M, pH 7.4) and contains CYP (25 pmoles/ml in the pre-incubation), NADPH (1 mM), substrate for the CYP being incubated and test inhibitor in DMSO (1% v/v). The substrate is prepared as detailed before (Automated TDI assay using HLM), but only a single substrate is used in this assay. The first stage of the assay is a serial dilution of the test inhibitor stocks to



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give five secondary solutions for each compound over a 50-fold concentration range. Following the serial dilution, CYP membranes (195 µl, 32 pmoles/ml) are added to the pre-incubation section of the incubation plate. Substrate (150 µl) and NADPH (50 µl, 5 mM) are added the secondary incubation section. The inhibitor solutions are then spiked (2.5 µl) into the pre-incubations and incubation is initiated by the addition of NADPH (52.5 µl, 5 mM NADPH). The plate is incubated at 37°C for 10 minutes. At this time point, aliquots (50 µl) of the 10 minute pre-incubates are transferred to secondary incubations containing substrate (150 µl) and NADPH (50 µl, 5 mM). After a further 10 minutes (20 minute pre-incubation), the procedure is repeated and finally the procedure is repeated for the 30 minute pre-incubates. The secondary incubations are allowed to proceed for 15 minutes. At this time aliquots (50 µl) are removed and added to the quench tubes containing methanol (100 µl). Quenched samples are chilled at –20$^{o}$C for 2 hours, centrifuged at 2000 g for 15 minutes and the supernatants transferred to microtitre plates for LC-MS-MS analysis.

**Manual $K_I$ and $k_{inact}$ Determinations for Standard Time Dependent Inhibitors of CYP3A4.**

Pre-incubations and secondary incubations were performed in 96-well microtitre plates. Wells contained recombinant CYP3A4 (195 µl, 32 pmoles) diluted from the stock (11 nmoles/ml) in phosphate buffer (0.1 M, pH 7.4). The wells also contained test inhibitor (2.5 µl at 100x the incubation concentration) in DMSO, or DMSO (2.5 µl) for solvent controls. The concentrations of the test inhibitors in the pre-incubation were: 4, 10, 20, 40, 100 and 200 µM (erythromycin); 0.05, 0.125, 0.25, 0.5, 1.25 and 2.5 µM (troleandomycin); 0.2, 0.5, 1, 2, 5 and 10 µM (mifepristone). A single plate was set up for each individual inhibitor investigated. The plates were pre-warmed for 3 minutes in an incubator at 37°C. Pre-warmed NADPH (52.5 µl, 5 mM), or phosphate buffer (52.5 µl) for zero time point samples, was the added to wells A1 – H1 and A2 – H2 respectively and the incubations continued. At the set time points of 0, 10, 30, 60, 150 and 300 seconds, aliquots (50 µl) were removed from the pre-incubation wells and added to wells containing pre-warmed NADPH (50 µl, 5 mM) and midazolam dose solution (150 µl, 16.7 µM). After a 10 minute incubation, aliquots from each well



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(50 µl) were removed and added to 1.2 ml polypropylene tubes containing cold methanol (100 µl). The samples were centrifuged and the samples analysed by LC-MS-MS.

**The Use of Testosterone as an Alternative CYP3A4 Substrate when Estimating $K_I$ and $k_{inact}$.**

For studying reversible inhibition of CYP3A4, it is now commonplace to use several different substrates, since different binding modes or sites within the enzyme's active site can give rise to different inhibition profiles. In order to directly compare midazolam and testosterone as substrates and to avoid non-specific binding to the enzyme preparation confounding the results, it was necessary to incubate identical CYP concentrations in the two assays. Since the rate of testosterone 6ß-hydroxylation is much lower than midazolam 1-hydroxylation, each pre-incubation contained 1250 pmoles CYP3A4 / ml. As a result of this high CYP3A4 concentration, the pre-incubation times were 2, 5 and 10 minutes. The secondary incubations where midazolam was the substrate were performed for only 2 minutes (according to time linearity studies performed using the elevated CYP3A4 concentration, data not shown). The secondary incubations with testosterone as the substrate were performed for 15 minutes. With the exception of these details, the assay was performed as described above (Manual $K_I$ and $k_{inact}$ Determinations for Standard Time Dependent Inhibitors).

**LC-MS-MS Analysis of Samples.**

LC-MS-MS analysis for the detection of paracetamol, 4-hydroxy diclofenac, 4-hydroxy S-mephenytoin, 1-hydroxy bufuralol and 1-hydroxy midazolam is performed as previously described (Weaver *et al*., 2003).

HPLC analysis and UV detection of 6ß-hydroxy testosterone was performed using a Waters 2795 separations module (Waters, UK), coupled to a Waters 996 Photodiode Array detector unit (Waters, UK). Samples (10 µl) was injected from a 96-well plate onto a Gemini (5 µm) C18 column (50 x 2 mm, Phenomenex, UK). Mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1 % formic acid in water), using a gradient of 5 % to 95 % (0 –



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2.5 min), 100 % A (2.51 – 3.0 min), 5 % A (3.05 min) with a run time of 4 min and flow rate 1 ml/min. 6ß-Hydroxy testosterone was detected by measuring the absorbance at 254 nm (retention time of 2.9 minutes), using an authentic standard as a reference.

**Analysis of the Data to Estimate $K_I$ and $k_{inact}$**

The natural log of the percent control activity remaining following incubation with a single inhibitor concentration is plotted against the time of pre-incubation (for example, see Figure 8). The slope, k, is the inactivation rate constant (describing the rate of inactivation at that inhibitor concentration). This is done for data from all inhibitor concentrations. Non linear regression analysis (WinNonlin$^{TM}$, Pharsight Corporation, North Carolina, USA) is then used to determine $K_I$ and $k_{inact}$ from the function

$$k = \frac{k_{inact} \cdot I}{K_I + I}$$

Where I is the pre-incubation inhibitor concentration

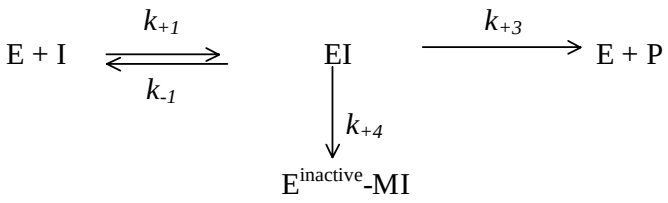
k is the inactivation rate constant for a given I

$k_{inact}$ is the maximal inactivation rate constant

$K_I$ is the inhibitor concentration, I, when the inactivation rate constant is half $k_{inact}$

**Analysis of the Data to Estimate Partition Ratio**

The partition ratio was determined according to the method described by Knight & Waley (1985) and Waley (1985) as follows:




E is the active enzyme



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I is the test inhibitor

P are the products of the reaction that escape reactive binding in the active site

EI is the initial complex formed between the enzyme and inhibitor

$E^{inactive}$-MI is the irreversible complex formed between the enzyme and the (irreversibly) bound reactive metabolite of the inhibitor.

The fate of the intermediate, EI, is governed by the partition ratio: the subsequent reaction can yield a metabolite that irreversibly binds to the enzyme or a metabolite that does not bind to the enzyme and escapes the active site. Accordingly, the partition ratio is the number of molecules of inhibitor (substrate) metabolised to product for each molecule that inactivates the enzyme (= $k_{+3} / k_{+4}$). (Partition ratio + 1) can be determined from the X-axis intercept of a plot of enzyme activity remaining (Y-axis) against inhibitor concentration / enzyme concentration (X-axis). This analysis can only provide a true partition ratio if all the inhibitor is metabolised. Often this plot is non-linear (Figure 1) and in these cases an estimation of the partition ratio can be best obtained by extrapolating to the X-axis intercept from the linear (low E/I ratio) portion of the plot. It must be remembered that under these circumstances the data provides only an estimate of partition ratio since inhibitor consumption may not be complete and the partition ratio may be over-estimated. The following equations demonstrate these points:

$$d[P]/dt = k_{+3} \,.\, [EI]$$

therefore

$$[P]_t = k_{+3} \,.\, \int_0^t [EI].dt$$

$$d[E^{inactive}]/dt = k_{+4} \,.\, [EI]$$

therefore

$$[E^{inactive}]_t = k_{+4} \,.\, \int_0^t [EI].dt$$

$[I]_0 = [E^{inactive}]_t + [P]_t$ if we assume $[I]_t = 0$ (i.e. all the inhibitor has been metabolised)

Where $[I]_0$ is the inhibitor concentration at time 0 and $[I]_t$ is the inhibitor concentration at time, t.



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It follows that:

$$[I]_0 = (k_{+4} + k_{+3}) \,.\, \int_0^t [EI].dt$$

At the X-axis intercept the enzyme activity remaining = 0

therefore $[E]_0 = [E^{inactive}]_t$

and it follows that

$$[E^{inactive}]_t = [E]_0 = k_{+4} \,.\, \int_0^t [EI].dt$$

Therefore,

$$[I]_0 / [E]_0 = \frac{(k_{+3} + k_{+4}).\ \int_0^t [EI].dt}{k_{+4}.\ \int_0^t [EI].dt}$$

Where the partition ratio = $k_{+3} / k_{+4}$

So (partition ratio + 1) = $[I]_0 / [E]_0$

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## Results

### Substrate Selectivity.

Recombinant human CYPs were individually incubated with the five CYP substrate cocktail (concentrations approximately equivalent to 4x Km). The CYP concentrations used were chosen to mimic those present in the human liver microsomal preparation used in the time dependent inhibition assay (Soars *et al.*, 2003). Under these conditions only CYP1A2 catalysed the phenacetin O-deethylation reaction, only CYP2C9 catalysed the diclofenac 4-hydroxylation reaction, only CYP2C19 catalysed the S-mephenytoin 4-hydroxylation reaction and only CYP3A4 metabolised midazolam to the 1-hydroxy metabolite. Additionally, the amount of metabolite formed was linear with respect to time over the 30 minute incubation period. CYP2D6 dependent bufuralol 1-hydroxylation was also linear with respect to time over the 30 minute incubation period. Bufuralol 1-hydroxylation was, however, catalyzed by CYP2C19 as well as CYP2D6. The HLM pool was assumed to contain 15 and 5 pmoles of CYP2C19 and CYP2D6 per mg microsomal protein respectively. Using these amounts of CYP in 1 ml incubations gave a CYP2C19 dependent rate of bufuralol 1-hydroxylation approximately equal to 40% of the CYP2D6 dependent bufuralol 1-hydroxylation.

### Selectivity of Time Dependent Inhibitors.

Time dependent inhibitors of CYP1A2 (furafylline), CYP2C9 (tienilic acid), CYP2C19 (ticlopidine), CYP2D6 (MDMA) and CYP3A4 (troleandomycin and mifepristone) were chosen as standard inhibitors to validate each run of the automated single time point, human liver microsomal time dependent inhibition assay. The inhibitors were apparently CYP-selective when incubated at 10 µM, 0.5 µM, 5 µM, 5 µM, 1.5 µM and 5 µM respectively (Table 1). The fact that the inhibitors were selective in the human liver microsomal assay confirms that the CYP-probe reactions themselves must be specific for the individual CYPs. For example, if the phenacetin O-deethylation reaction was performed by CYP2C19 as well as CYP1A2, a time dependent inhibition of the reaction would have been observed when ticlopidine was used as an inhibitor. It would therefore have appeared as if ticlopidine was an inhibitor of CYP1A2. The CYP2C19 time dependent inhibitor ticlopidine did not show a



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strong inhibition of bufuralol 1-hydroxylase activity, indicating that the lack of selectivity of this probe reaction does not impact significantly on the interpretation of the results using this HLM pool in the single time point HLM TDI assay (Table 1).

**Time Dependent (Irreversible) $IC_{50}$ Determination using the Automated Single Time Point, Human Liver Microsomal Time Dependent Inhibition Assay.**

Furafylline, tienilic acid, ticlopidine, MDMA, troleandomycin and mifepristone were separately incubated at five concentrations, using the automated single time point human liver microsomal assay. The results (Figure 2) demonstrate that each inhibitor decreased the control activity approximately 80% (from 90% to 10%) over an inhibitor concentration range of 2 log units (100-fold). Therefore a 1% change in control activity is brought about by a 0.025 log unit change in inhibitor concentration. Assuming this to be true for all time dependent inhibitors, equation 1 was written to project an $IC_{50}$ value from a single inhibitor concentration and percent inhibition relative to control.

$$\text{Projected } IC_{50} = \text{inhibitor concentration} \times 10^{(50 - \%\text{ time dependent inhibition}) \times 0.025} \quad (1)$$

where the % time dependent inhibition is calculated as follows:

$$\%\,TDI = 1 - \frac{R+I^{NADPH} / R-I^{NADPH}}{R+I^{NO\ NADPH} / R-I^{NO\ NADPH}} \times 100 \quad (2)$$

$R+I^{NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the presence of inhibitor and NADPH

$R-I^{NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the absence of inhibitor (i.e. solvent control) but presence of NADPH

$R+I^{NO\ NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the presence of inhibitor but absence of NADPH



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$R\text{-}I^{NO\ NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the absence of inhibitor (i.e. solvent control) and NADPH

$IC_{50}$ values for all the standard inhibitors are shown in Table 1.

**$K_I$ and $k_{inact}$ Determinations for Known Time Dependent Inhibitors Using the Automated Three Time Point, Six Concentration, Recombinant CYP TDI Assay**

The automated TDI assay was used to generate kinetic parameters for time dependent CYP inhibitors selected from the literature. An example of the data generated is shown in Figure 3. The comparison with $k_{inact}$ and $K_I$ values taken from the literature is presented in Table 2. Generally there is good agreement between our values and those quoted in the literature. The only literature $k_{inact}$ and $K_I$ values available for tienilic acid and ticlopidine were generated in the same laboratory using microsomes prepared from yeast expressing human CYP. The expression levels of CYP2C9 and CYP2C19 per mg microsomal protein were very low and the experiments consequently used high microsomal protein concentrations. This may be the reason why the $k_{inact}$ values from our assay are similar to the literature values, but the $K_I$ values we report are lower.

**Manual $K_I$ and $k_{inact}$ Determinations for Standard Time Dependent Inhibitors**

Three of the time dependent CYP3A4 inhibitors profiled in the automated assay were chosen for manual $k_{inact}$ and $K_I$ determination. The manual assay was set up to replicate the automated assay in all features except that the pre-incubation times were 0, 10, 30, 60, 150 and 300 seconds. The purpose of this was to investigate the appropriateness of the 10, 20, 30 minute pre-incubation times used in the automated assay. The results (Table 3) show that the automated assay conditions are effective for robust and accurate $K_I$ and $k_{inact}$ determinations.

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**The Use of Testosterone as an Alternative CYP3A4 Substrate when Estimating $K_I$ and $k_{inact}$.**

The high CYP3A4 concentrations used for this comparison meant that the pre-incubation times had to be short (2, 5, 10 minutes) and the secondary incubation with midazolam was only two minutes (in order to maintain time-linearity of the 1-hydroxy metabolite formation). Data presented here suggest that there is little difference in the $K_I$ and $k_{inact}$ values determined using testosterone and midazolam as CYP3A4 substrates for those compounds tested (Table 3).

**Relationship Between Time Dependent $IC_{50}$ Determined Using the Automated Single Time Point Human Liver Microsomal Assay and $k_{inact}$ / $K_I$ Determined Using the Automated Three Time Point, Six Concentration Recombinant CYP Assay**

In order to put results from the initial HLM assay into context, a relationship between the irreversible $IC_{50}$ and the kinetic constants of time dependent inhibition ($k_{inact}$, $K_I$) needs to exist. Since increasing $IC_{50}$ values are associated with decreasing potency of inhibition, the reciprocal of the unbound $IC_{50}$ values were plotted against $k_{inact}/K_I$ (a measure of the efficiency of CYP inactivation) for twenty eight compounds shown to be time dependent inhibitors of CYP3A4. The unbound $IC_{50}$ value was calculated by multiplying the $IC_{50}$ value by the fraction of inhibitor unbound in the 1 mg/ml hepatic microsomal pre-incubation ($fu_{inc}$, calculated from the equation described by (Austin *et al.*, 2002)). $Fu_{inc}$ for the inhibitor in the automated three time point, six concentration recombinant CYP assay was always assumed to 1. Figure 4 demonstrates that the initial HLM screen is effective in providing an estimate of $k_{inact}$ / $K_I$.

**Relationship Between $k_{inact}$/ $K_I$ and Partition Ratio**

The partition ratio is sometimes used to express the efficiency of enzyme inactivation, since it describes the number of molecules of inhibitor (substrate) metabolised to product for each molecule that inactivates the enzyme. The data generated in the automated time dependent



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inhibition assay, from twenty eight compounds shown to time dependent inhibitors of CYP3A4 were used to determine partition ratio, $K_I$ and $k_{inact}$. Figure 5 shows that a good relationship exists between $k_{inact}/K_I$ and partition ratio. The equation of the line of best fit shows that for this data set, a partition ratio of 10 is equivalent to a $k_{inact}/K_I$ ratio of 0.79 ml/min.nmole.

**TDI Screening Strategy**

Since the initial HLM screen is effective in providing an estimate of $k_{inact}/K_I$, which is itself closely related to the partition ratio as a measure of TDI potency, a proposed screening strategy is to use the higher throughput HLM assay to generate an $IC_{50}$ for assessment of the pharmacokinetic impact on co-therapies using the estimated $k_{inact}/K_I$ ratio. When necessary, for compounds where a DDI is deemed likely, $k_{inact}$ and $K_I$ values can be determined accurately using the single CYP automated assay. This paradigm is depicted in Figure 6.

**Use of the Reversible Inhibition Data Obtained from the TDI Assays as '*Quality Control Measures*'**

Both the TDI assays are designed to discriminate between reversible and irreversible CYP inhibition. Compounds are screened in the reversible CYP inhibition assay (described by Weaver *et al*., 2003) prior to incubation in the TDI assays and reversible inhibition $IC_{50}$ values can therefore be used to '*quality control*' and cross-check the data from the TDI assays. The following sections describe how these checks are made.

**Reversible $IC_{50}$ Determination Using the Automated Single Time Point, Human Liver Microsomal Time Dependent Inhibition Assay**

The assay was designed to discriminate between reversible and irreversible CYP inhibition since the pre-incubation step is performed in the presence and absence of NADPH. $IC_{50}$ values for any reversible inhibition detected can be calculated in a similar way to that described for irreversible time dependent inhibitors (equation 1), since reversible inhibitors



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decrease control activity from 90% to 10% over an inhibitor concentration range of 2 log units (Segel, 1975).

$$\text{Projected } IC_{50} = (1/5 \times \text{initial inhibitor concentration}) \times 10^{(50 - \% \text{ reversible inhibition}) \times 0.025} \quad (3)$$

where the % reversible inhibition is calculated as follows:

$$\% \text{ reversible inhibition} = \frac{R+I^{NO\ NADPH}}{R-I^{NO\ NADPH}} \quad (4)$$

$R+I^{NO\ NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the presence of inhibitor but absence of NADPH

$R-I^{NO\ NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the absence of inhibitor (i.e. solvent control) and NADPH

The 5-fold dilution factor is included in equation 3 because an aliquot of the initial pre-incubation (50 µl) is diluted into the final incubation with the CYP probe substrates (250 µl). This is a key difference compared to determining $IC_{50}$ values for the irreversible, time dependent CYP inhibition (equation 1). It is necessary because the reversible CYP inhibition occurs in the second incubation, whereas the time dependent inhibition occurs in the pre-incubation, when inhibitor concentrations are 5-fold higher.

In order to compare the reversible CYP inhibition $IC_{50}$ values calculated in this way with $IC_{50}$ values generated from the reversible CYP inhibition screen (Weaver *et al.*, 2003), the discrepancy in substrate concentration between the two assays has to be taken into account (CYP probe substrate is equal to Km in the reversible CYP inhibition screen but approximately equal to 4x Km in the time dependent inhibition assay). Therefore, since a competitive $IC_{50}$ is equal to Ki (1 + [S]/Km), the following relationship should exist:

$$IC_{50} \text{ value } 2 = 2.5 \times IC_{50} \text{ value } 1 \quad (5)$$



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Where $IC_{50}$ value 1 is estimated from the normal reversible CYP inhibition screen and $IC_{50}$ value 2 is the reversible inhibition $IC_{50}$ estimated from the time dependent inhibition assay. Accounting for non-specific binding differences (due to the fact that the reversible CYP inhibition screen uses *E. coli* membrane preparations containing recombinant human CYP and the assay described here uses HLM) was deemed to be largely unimportant, since the secondary incubation of the TDI assay contains only 0.2 mg/ml microsomal protein. Obviously the relationship described in equation 5 does not hold for non-competitive inhibitors, where $IC_{50}$ is independent of substrate concentration. Figure 7 shows that there is a good correlation between the $IC_{50}$ values determined in both assays. This type of interrogation of the data generated in the TDI screen acted as a quality control of the assay.

**The Effect of Reversible CYP Inhibitors in the Automated Single Time Point, Human Liver Microsomal Time Dependent Inhibition Assay.**

To demonstrate that the automated human liver microsomal time dependent inhibition screen could discriminate between reversible and irreversible CYP inhibition, potent reversible inhibitors of CYP2C9 (sulphaphenazole 1 and 10 μM) CYP2D6 (quinidine 0.025 and 0.25 μM) and CYP3A4 (ketoconazole 0.005 and 0.05 μM), were incubated. Sulphaphenazole reduced the diclofenac 4-hydroxylation activity by the same amount when pre-incubations were performed in the presence and absence of NADPH, indicating reversible but not time dependent inhibition. The same was true for quinidine (inhibition of bufuralol 1-hydroxylation) and ketoconazole (inhibition of midazolam 1-hydroxylation). Equation 3 was used to calculate reversible $IC_{50}$ values from the single inhibitor concentrations used. These values compare well to $IC_{50}$ values generated in the reversible CYP inhibition screen (Table 4).

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**Reversible $IC_{50}$ Determination Using Data From the Automated Three Time Point, Six Concentration, Recombinant CYP TDI Assay**

The data from this assay are used to generate $K_I$ and $k_{inact}$ values (described in Materials and Methods). The first step is to plot the natural log of the percent control activity remaining, following incubations with a range of inhibitor concentrations, against the time of pre-incubation (Figure 8). It is obvious from the data that the straight lines of best fit do not always back-extrapolate to 4.6 (i.e. 100% control activity), even when very short incubation times are used. Clearly the back-extrapolated percent control activity represents the inhibition that occurs when no pre-incubation has taken place. This inhibition may be a mixture of reversible and irreversible inhibition occurring during the final 15 minute co-incubation with CYP substrate. Thus, the time zero percent control activity for a given inhibitor concentration should be equivalent to the percent control activity determined in the reversible CYP inhibition screen described previously (Weaver *et al.*, 2003). As detailed in Figure 8, these time zero percent control activities can be used with the respective inhibitor concentrations (once the 5-fold dilution from the pre-incubation had been accounted for) to determine an $IC_{50}$ value for the reversible inhibition component. Table 3 shows that the data from the time dependent inhibition assay match well with the data generated in the reversible CYP inhibition screen. A good fit of the ln percent control activity / time plot coupled with this check on the Y-intercept values serve as valuable quality control assessments for the assay. Whilst this exercise offers a way to compare the CYP inhibition data from two different assays, it is not ideal to have extensive reversible inhibition in the TDI assay. Clearly 10-fold or greater dilution factors from the first to the second incubation in the TDI assay can reduce the impact of this complication.

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**Discussion**

Many pharmaceutical companies utilise microtitre plate methods with real time detection of fluorescent metabolites to facilitate high throughput screening for reversible CYP inhibition. A simple modification to this method facilitates an early time dependent inhibition screen (detection of a time dependent shift in $IC_{50}$) (Naritomi *et al.*, 2004;Yamamoto *et al.*, 2002). Whilst large numbers of compounds can be studied with such methodology, interpretation of the $IC_{50}$ data may be confounded if the opposing effects of TDI (decreasing $IC_{50}$) and significant depletion of inhibitor concentration (increasing the reversible inhibition $IC_{50}$) are manifested. Furthermore, since several reactions may contribute to many TDI episodes (Bensoussan *et al.*, 1995), it is conceivable that one particular CYP may be involved in generating metabolites that are then further metabolised to reactive species in the active site of a second CYP. Since the fluorescent probes used in these assays are generally not CYP-specific, the experiments necessitate the use of single CYPs, rather than HLM or CYP cocktails. It is therefore not inconceivable that false negative results could occur. Moreover, the suitability of fluorescent probes for making *in vivo* projections from *in vitro* reversible CYP inhibition experiments has recently been challenged (Cohen *et al.*, 2003). Industry guidelines for *in vitro* drug-drug interaction studies recommend the use of CYP probe substrates that may be said to be more “drug-like”, although the list of substrates was not chosen with this specific intention (Tucker *et al*., 2001).

Recently, this laboratory implemented a reversible CYP inhibition assay using a cocktail of the recommended CYP1A2, 2C9, 2C19, 2D6 and 3A4 substrates at concentrations equivalent to their respective Km values. LC-MS-MS was used to follow the formation of specific metabolites (Weaver *et al.*, 2003). The same end-point was used in the assays described here, although the substrate concentrations were approximately four-times their respective Km values. Analysis may be less rapid than with fluorescence-based assays and the initial time dependent inhibition assay described here does not easily afford screening on a large scale. However, given the fact that the functional groups and mechanisms involved in the majority



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of irreversible CYP inhibition cases are well known (Ortiz de Montellano & Correia, 1995; Bensoussan *et al.*, 1995; Franklin, 1977), compounds can be rationally chosen based on structural features and, in order to avoid overlooking potentially inhibitory compounds, how well they represent chemistry that is current within a project.

In order to determine the CYP-selectivity of the reactions in the HLM incubations using the elevated substrate concentrations, individual CYPs were incubated with each substrate. CYP concentrations were chosen based on a calculation of the likely concentration in the HLM pool. Under these conditions, the five reactions were deemed to be appropriate CYP-selective reactions for the single time point HLM assay. Bufuralol 1-hydroxylation was catalyzed by CYP2C19 at a rate approximately equal to 40% of the CYP2D6 catalysed reaction. In this case, CYP2D6 would only account for 70% of the total bufuralol 1-hydroxylase activity and, in theory, the potency of a CYP2D6 time dependent inhibitor could be under-estimated by a factor of three. However, studies with the CYP2C19 time dependent inhibitor ticlopidine indicated that the lack of CYP-selectivity of the bufuralol 1-hydroxylation reaction may not impact significantly on the interpretation of the TDI results.

The assay for determining $K_I$ and $k_{inact}$ for a single recombinant CYP uses three time points. Ideally the assay would use more time points and ensure that these adequately span the inactivation half-life. However, a pragmatic approach of six inhibitor concentrations pre-incubated for 10, 20 and 30 minutes was adopted to reduce sample numbers. In order to validate this automated approach, more comprehensive manual assays were performed and the TDI kinetic parameters generated were compared to those generated from the three time-point automated assay. This comparison demonstrated the effectiveness of the automated assay for describing the enzyme inactivation by inhibitors with a range of $k_{inact}/K_I$ values. The $k_{inact}$ and $K_I$ values determined in the automated TDI assay described here compare well with previously published results (Table 2). An additional concern was the capability of the assay to generate data uncontaminated by reversible CYP inhibition. Both the automated assays described here use a five-fold dilution of the pre-incubation into the final incubation with the

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CYP probe substrates, whereas ten-fold or greater dilutions are commonly used. Figure 8 clearly demonstrates that the 5-fold dilution can give rise to concentrations of inhibitor that give considerable reversible inhibition in the second incubation. This should not greatly alter the slopes of natural log percent control activity – time plots and, therefore, should not alter the kinetic parameters of inhibition estimated. However, the slopes may be easier to determine if the percentage inhibition is not swamped by the reversible CYP inhibition and therefore it is advisable to use a 10-fold or greater dilution factor if sufficient analytical sensitivity (detecting 1-hydroxy midazolam formation) can be achieved. This is of particular importance when test inhibitors are reasonably potent reversible CYP inhibitors but moderate to weak time dependent inhibitors.

It is now commonplace to use several different substrates for reversible CYP3A4 inhibition assays, since different substrate binding sites within the active site of the enzyme can confound *in vivo* projections (Kenworthy *et al.*, 1999). The majority of TDI studies use a pre-incubation step involving inhibitor and enzyme followed by the addition of substrate once the enzyme has been inactivated. This experimental design suggests that data for different CYP3A4 substrates would be comparable if inactivation occurs as a result of haem binding. Substrate differences have been demonstrated for time dependent inhibition studies, using midazolam as a time dependent inhibitor (Schrag and Wienkers, 2001). However, midazolam oxidation results in protein adduct formation in the CYP3A4 active site, which could disparately influence the binding of diverse substrates. The substrate comparison described here was not straightforward, due to the very different rates of metabolism of the two substrates - a high concentration of enzyme was required (1250 pmoles/ml) for the testosterone incubations. To facilitate the comparison by avoiding differences in non-specific binding, the same enzyme concentration was used for the assay when midazolam was used as a substrate (a forty-fold increase in CYP3A4 concentration over the standard time dependent inhibition assay). Short pre-incubation times and a short incubation with midazolam were therefore required. There was little difference in the $K_I$ and $k_{inact}$ values determined using testosterone and midazolam as CYP3A4 substrates for the compounds studied. It is interesting



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to compare the results from this experiment, when midazolam was used as a CYP3A4 substrate, with the results from the standard automated assay for $k_{inact}$ and $K_I$ determination using midazolam as a substrate. The $k_{inact}$ values determined for mifepristone in the two assays were very similar (0.08 $min^{-1}$ and 0.07 $min^{-1}$). The same was found to be true for erythromycin (0.12 $min^{-1}$ and 0.09 $min^{-1}$) and troleandomycin (0.12 $min^{-1}$ and 0.16 $min^{-1}$). The $K_I$ values for mifepristone were 0.6 µM and 2.8 µM, for erythromycin 8.8 µM and 8.3 µM and troleandomycin 0.3 µM and 0.8 µM. The differences in logP and therefore non-specific binding most likely account for differences in observed (apparent) $K_I$ (Austin *et al.*, 2002).

The results of the studies detailed above demonstrate that both automated assays are appropriate for the generation of creditable and robust data on the time dependent inhibitory potency of test compounds. The potent reversible inhibitors sulphaphenazole, ketoconazole and quinidine did not cause TDI, but significant reversible inhibition was detected. The $IC_{50}$ values estimated for this reversible inhibition component (albeit projected off single inhibitor concentrations, equation 3) matched well with those determined in the separate reversible CYP inhibition screen (Weaver *et al*., 2003). This has also consistently been shown to be true for a range of compounds that have been assayed in both screens (Figure 7) and serves as a good quality control check for each experiment.

$IC_{50}$ values are generated for the TDI component (equation 1) but because HLM are used in this initial assay, the $IC_{50}$ values can only be treated as apparent values. Unbound $IC_{50}$ values are calculated using a lipophilicity term (logP for bases, logD for acid and neutral compounds) to estimate the extent of non-specific binding *in vitro* (Austin *et al*., 2002). Despite the single time point of 30 minutes used in the initial screen, a good relationship exists between the unbound $IC_{50}$ estimated in this format and the $k_{inact}/K_I$ derived from the more extensive subsequent single CYP assay. This means that the initial screening assay generates an inhibitory potency term ($IC_{50}$) that has a meaningful value. The $k_{inact}/K_I$ itself was shown to relate well to the partition ratio as a measure of inhibitory potency or efficiency



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of enzyme inactivation (Figure 5). The screening strategy proposed would therefore use the higher throughput HLM assay to generate an $IC_{50}$ for assessment of the pharmacokinetic impact on co-therapies using the estimated $k_{inact}/K_I$ ratio, predicted human dose and pharmacokinetic parameters (clearance, volume of distribution and plasma protein binding data). This scheme is depicted in Figure 6. When necessary, for compounds where a DDI is deemed likely, $k_{inact}$ and $K_I$ values can be determined accurately using the single CYP automated assay.

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Figure 1. Plot of remaining enzyme activity against inhibitor concentration / enzyme concentration for troleandomycin and recombinant CYP3A4. The data were generated using the automated three time point, six inhibitor concentration, time dependent inhibition assay, with midazolam 1-hydroxylation as a measure of CYP3A4 activity. The dashed line is the regression line used to estimate the partition ratio from the X-axis intercept (see Materials and Methods)

Figure 2. Inhibition of the CYP probe reactions, (A) phenacetin O-deethylation (CYP1A2), (B) diclofenac 4-hydroxylation (CYP2C9), (C) S-mephenytoin 4-hydroxylation (CYP2C19), (D & E) midazolam 1-hydroxylation (CYP3A4) and (F) bufuralol 1-hydroxylation (CYP2D6) by standard time dependent inhibitors in the single pre-incubation time point (30 minutes), human liver microsomal time dependent inhibition assay

Figure 3. Plot of inactivation rate constant (k) and inhibitor concentration (I) for troleandomycin. The data were generated using recombinant human CYP3A4 in the automated time dependent inhibition assay. The natural log of the percent control activity remaining following incubation with each inhibitor concentration was plotted against the time of pre-incubation. The slopes, k, (the inactivation rate constants) were used with inhibitor concentration to determine $K_I$ and $k_{inact}$ by non-linear regression analysis applying the equation

$k = (k_{inact} . I) / (I + K_I)$.

Figure 4. Plot of log 1/ unbound $IC_{50}$ against log $k_{inact}/K_I$ for twenty eight time dependent inhibitors of CYP3A4. $IC_{50}$ values were generated using the automated single time point, single inhibitor concentration human liver microsomal time dependent inhibition screen. These values were adjusted for incubational binding to give unbound $IC_{50}$ values (according to the mathematical model of Austin et al., 2002). $K_I$ and $k_{inact}$ were estimated using the three time point, six inhibitor concentration, recombinant CYP automated time dependent inhibition assay. The line of best fit is shown with dashed lines representing three-fold deviation from the line.

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Figure 5. Plot of log partition ratio against log $k_{inact}/K_I$ for twenty eight time dependent inhibitors of CYP3A4. Data were generated using the three time point, six inhibitor concentration, recombinant CYP automated time dependent inhibition assay.

Figure 6. A general paradigm for TDI screening using the two automated assays described in the text. Since both assays differentiate between reversible and irreversible CYP inhibition, the reversible inhibition data from the TDI assays can be cross-checked with data from the standard reversible CYP inhibition screen (described by Weaver et al., 2003) as a 'quality control' measure for the TDI assay data.

Figure 7. Comparison of reversible inhibition $IC_{50}$ values (μM) generated in the reversible CYP inhibition screen (set up as described in Weaver et al., 2003) and reversible inhibition $IC_{50}$ values projected from the single concentration human liver microsomal time dependent inhibition screen. These $IC_{50}$ values are calculated according to equations 3 - 5 (Results).

Figure 8. Inhibition of recombinant CYP3A4 dependent midazolam 1-hydroxylation by the time dependent inhibitor erythromycin (EMC). The assay was performed manually using EMC pre-incubation concentrations of 4 – 200 μM, as described in Materials and Methods. Zero minute pre-incubation time point samples were removed and added to the secondary incubation with NADPH and midazolam (10 μM). The formation of 1-hydroxy midazolam was monitored. The percentage midazolam 1-hydroxylation activity relative to control (no EMC present in the pre-incubation) was plotted against EMC concentration in the secondary incubation (1/5 x the pre-incubation concentrations) and an $IC_{50}$ value estimated.

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TABLE 1. Inhibition of the CYP probe reactions, phenacetin O-deethylation (POD), diclofenac 4-hydroxylation (D4OH), S-mephenytoin 4-hydroxylation (SM4OH), bufuralol 1-hydroxylation (B1OH) and midazolam 1-hydroxylation (M1OH) by standard time dependent inhibitors. Percentage TDI (time dependent inhibition) was calculated from the ratio of $R+I^{NADPH}$ / $R-I^{NADPH}$ and $R+I^{NO\ NADPH}$ / $R-I^{NO\ NADPH}$ as detailed in Results (Equation 2). Projected $IC_{50}$ was calculated according to equation 1 (Results). Inhibitor concentrations were as follows: Furafylline (FUR), 10 µM; tienilic acid (TA), 0.5 µM; ticlopidine (TIC), 5 µM; MDMA, 5 µM; troleandomycin (TAO), 1.5 µM; mifepristone (MIF), 5 µM. Data are the mean of thirty experiments. For each inhibitor, the coefficient of variance (CV) was less than 5% for %TDI values and less than 20% for projected $IC_{50}$ values.

| Probe reaction | Inhibitor | $R+I^{NADPH}$ / $R-I^{NADPH}$ | $R+I^{NO\ NADPH}$ / $R-I^{NO\ NADPH}$ | % TDI (mean±SD) | Projected $IC_{50}$ (µM) |
|---|---|---|---|---|---|
| POD | FUR | 0.16 | 0.75 | 79 ± 3 | 1.9 |
| | TA | 0.87 | 0.98 | 6 ± 3 | |
| | TIC | 0.91 | 0.90 | 0 | |
| | MDMA | 0.95 | 0.92 | 0 | |
| | TAO | 0.93 | 1 | 6 ± 2 | |
| | MIF | 0.94 | 0.94 | 0 | |
| D4OH | FUR | 0.91 | 0.87 | 0 | |
| | TA | 0.32 | 0.8 | 66 ± 6 | 0.2 |
| | TIC | 0.94 | 0.93 | 0 | |
| | MDMA | 0.94 | 0.89 | 0 | |
| | TAO | 0.93 | 0.99 | 6 ± 1 | |
| | MIF | 0.81 | 0.83 | 3 ± 1 | |
| SM4OH | FUR | 0.91 | 0.94 | 4 ± 2 | |
| | TA | 0.96 | 1 | 8 ± 3 | |
| | TIC | 0.29 | 0.63 | 54 ± 9 | 3.9 |
| | MDMA | 0.99 | 0.99 | 0 | |
| | TAO | 0.91 | 0.96 | 6 ± 3 | |
| | MIF | 0.96 | 0.96 | 0 | |
| B1OH | FUR | 0.96 | 0.89 | 0 | |
| | TA | 0.98 | 0.96 | 0 | |
| | TIC | 0.96 | 0.94 | 0 | |
| | MDMA | 0.26 | 0.77 | 67 ± 2 | 1.9 |
| | TAO | 0.85 | 0.9 | 6 ± 1 | |
| | MIF | 0.89 | 0.87 | 0 | |
| M1OH | FUR | 0.99 | 0.93 | 0 | |
| | TA | 1.01 | 1.00 | 0 | |
| | TIC | 1.00 | 1.02 | 0 | |
| | MDMA | 1.01 | 0.94 | 0 | |
| | TAO | 0.28 | 0.9 | 69 ± 3 | 0.5 |
| | MIF | 0.33 | 0.71 | 53 ± 4 | 4.1 |

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TABLE 2. Comparison of literature values for $K_I$ and $k_{inact}$ with values estimated from data generated using the automated time dependent inhibition assay described in this publication.

| | CYP | $K_I$ (μM) | $k_{inact}$ ($min^{-1}$) | Literature $K_I$ (μM) | Literature $k_{inact}$ ($min^{-1}$) | References |
|---|---|---|---|---|---|---|
| Troleandomycin | 3A4 | 0.26 | 0.121 | 0.18 – 0.73 | 0.15 – 0.27 | 1, 2, 3 |
| Mifepristone | 3A4 | 0.61 | 0.080 | 0.5 - 4.7 | 0.089 – 0.18 | 3, 4 |
| Erythromycin | 3A4 | 8.82 | 0.120 | 5 - 82 | 0.011 – 0.173 | 2, 5, 6, 7, 8, 9 |
| Diltiazem | 3A4 | 0.49 | 0.010 | 2 | 0.015 – 0.17 | 1, 3, 8, 10 |
| Verapamil | 3A4 | 0.74 | 0.041 | 1.7 – 5.9 | 0.09 – 1.1 | 1, 3, 11 |
| Ethinylestradiol | 3A4 | 23 | 0.09 | 18 | 0.04 | 12 |
| Tienilic acid | 2C9 | 0.85 | 0.23 | 4.3 | 0.2 | 14 |
| MDMA | 2D6 | 6.4 | 0.23 | 13 | 0.29 | 13 |
| Ticlopidine | 2C19 | 9.2 | 0.25 | 87 | 0.19 | 14 |

(1) Zhou et al., 2005; (2) Chan & Delucchi 2000; (3) McCabe et al., 2002; (4) He et al., 1999; (5) Ito et al., 2003; (6) McConn et al., 2004; (7) Kanamitsu et al., 2000; (8) Zhou et al., 2005; (9) Yamano et al., 2001; (10) Jones, 1999; (11) Wang, 2004; (12) Lin et al., 2002; (13) Heydari et al., 2004; (14) Ha-Duong et al., 2001

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TABLE 3. Comparison of the kinetic constants of reversible and time dependent inhibition (TDI) determined from manual and automated assays for troleandomycin (TAO), mifepristone (MIF) and erythromycin (EMC). The reversible inhibition $IC_{50}$ values derived from the TDI data were calculated as described in Figure 8. The reversible inhibition screen was performed as described by Weaver *at al*., 2003. Manual and automated experiments were n = 3 with CV values less than 10% for $K_I$ and $k_{inact}$.

| | Substrate | TAO | MIF | EMC |
|---|---|---|---|---|
| Manual $k_{inact}$ ($min^{-1}$) | Midazolam* | 0.25 | 0.09 | 0.16 |
| Automated $k_{inact}$ ($min^{-1}$) | Midazolam* | 0.12 | 0.08 | 0.12 |
| Manual $K_I$ (µM) | Midazolam* | 0.47 | 0.85 | 9.5 |
| Automated $K_I$ (µM) | Midazolam* | 0.26 | 0.61 | 8.8 |
| Manual $k_{inact}$ ($min^{-1}$) | Midazolam** | 0.16 | 0.07 | 0.09 |
| Manual $k_{inact}$ ($min^{-1}$) | Testosterone** | 0.06 | 0.06 | 0.06 |
| Manual $K_I$ (µM) | Midazolam** | 0.8 | 2.8 | 8.3 |
| Manual $K_I$ (µM) | Testosterone** | 0.7 | 4.1 | 9.5 |
| Reversible $IC_{50}$ derived from the TDI experiment (µM) | Midazolam | 0.3 | 1.5 | 8 |
| $IC_{50}$ value estimated from the reversible CYP inhibition screen (µM) | Midazolam | 0.5 | 0.7 | 8 |

** Incubations contained 1250 pmoles CYP3A4 / mL

* Incubations contained 25 pmoles CYP3A4 / mL

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TABLE 4. Comparison of reversible $IC_{50}$ values generated from the reversible CYP inhibition screen (Weaver *et al.*, 2003) for sulphaphenazole (SPZ), quinidine (QUI) and ketoconazole (KCZ) and those projected from a single inhibitor concentration in the human liver microsomal, single time point time dependent inhibition assay.

| | $R+I^{NADPH}$ / $R-I^{NADPH}$ | $R+I^{NO\ NADPH}$ / $R-I^{NO\ NADPH}$ | % TDI[a] | Projected reversible $IC_{50}$ [b] (μM) | $IC_{50}$ [c] (μM) |
|---|---|---|---|---|---|
| 1 μM SPZ | 0.61 | 0.59 | -4.5 | 0.14 | 0.2 |
| 10 μM SPZ | 0.19 | 0.18 | -5.1 | 0.13 | 0.2 |
| 25 nM QUI | 0.81 | 0.83 | 1.9 | 0.013 | 0.015 |
| 250 nM QUI | 0.49 | 0.54 | 8.2 | 0.022 | 0.015 |
| 5 nM KCZ | 0.97 | 0.92 | -5 | 0.005 | 0.005 |
| 50 nM KCZ | 0.87 | 0.8 | -8 | 0.028 | 0.005 |

$R+I^{NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the presence of inhibitor and NADPH

$R-I^{NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the absence of inhibitor (i.e. solvent control) but presence of NADPH

$R+I^{NO\ NADPH}$ is the rate of CYP probe reaction when the initial (pre-) incubation is performed in the presence of inhibitor but absence of NADPH

[a] Percentage time dependent inhibition (TDI) calculated according to equation 2 (Results). Values are the average of duplicate incubations performed within a single experiment.

[b] Projected reversible $IC_{50}$ calculated according to equations 3 - 5 (Results). Values are the average of duplicate incubations performed within a single experiment.

[c] Reversible $IC_{50}$ values generated from the reversible CYP inhibition screen (set up as described in Weaver *et al.*, 2003). Values are the mean of 150 replicates.

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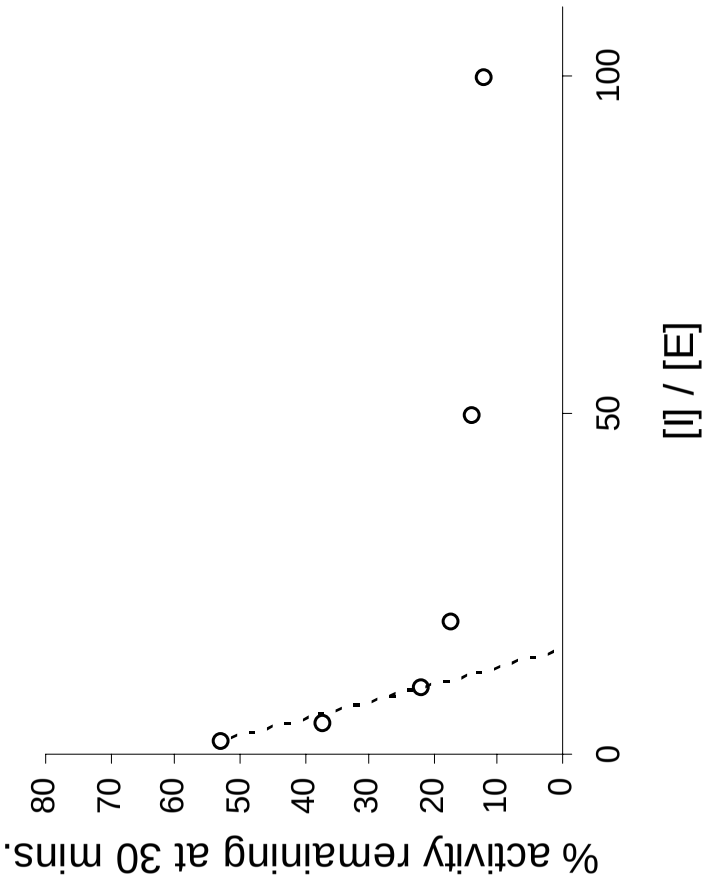
Figure 1

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# Figure 2

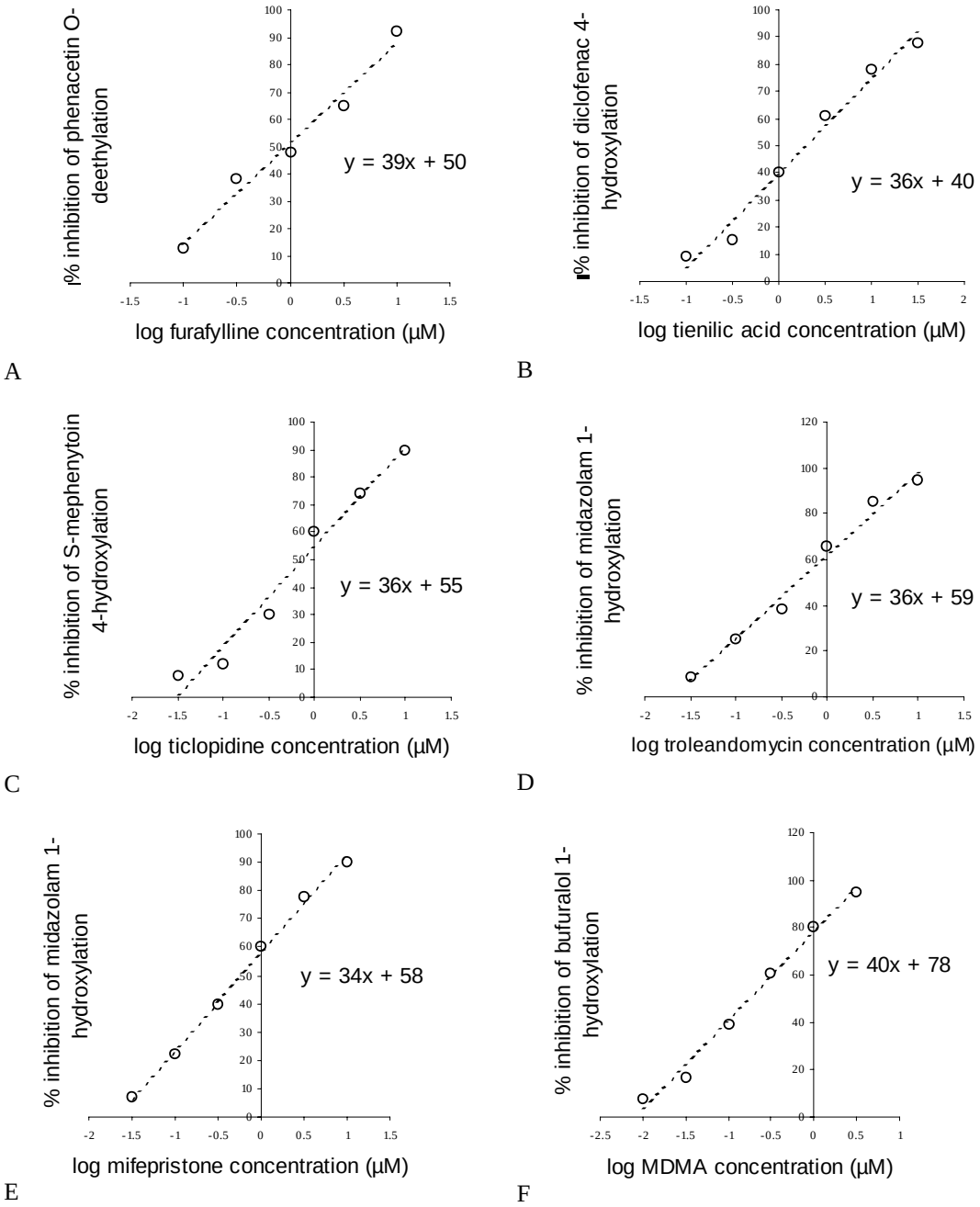


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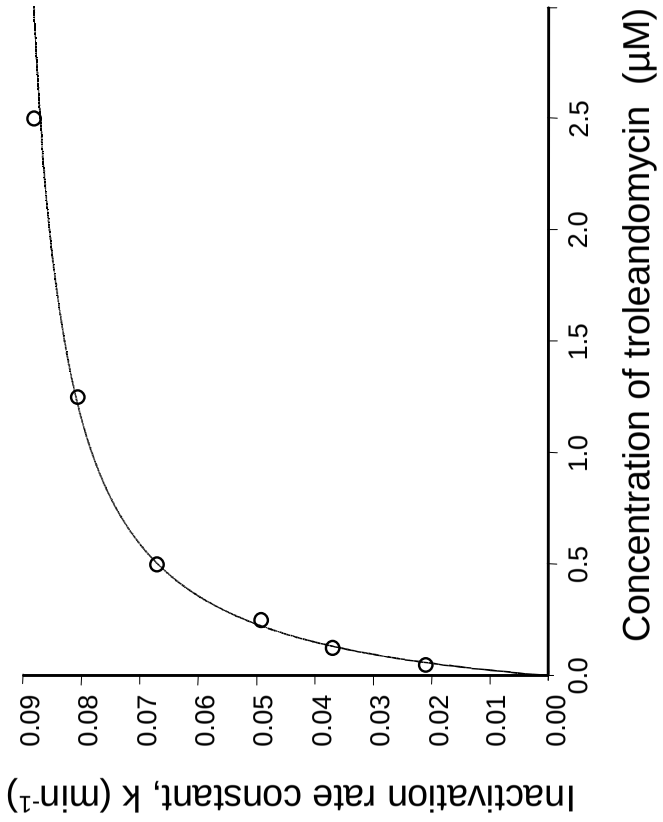
Figure 3

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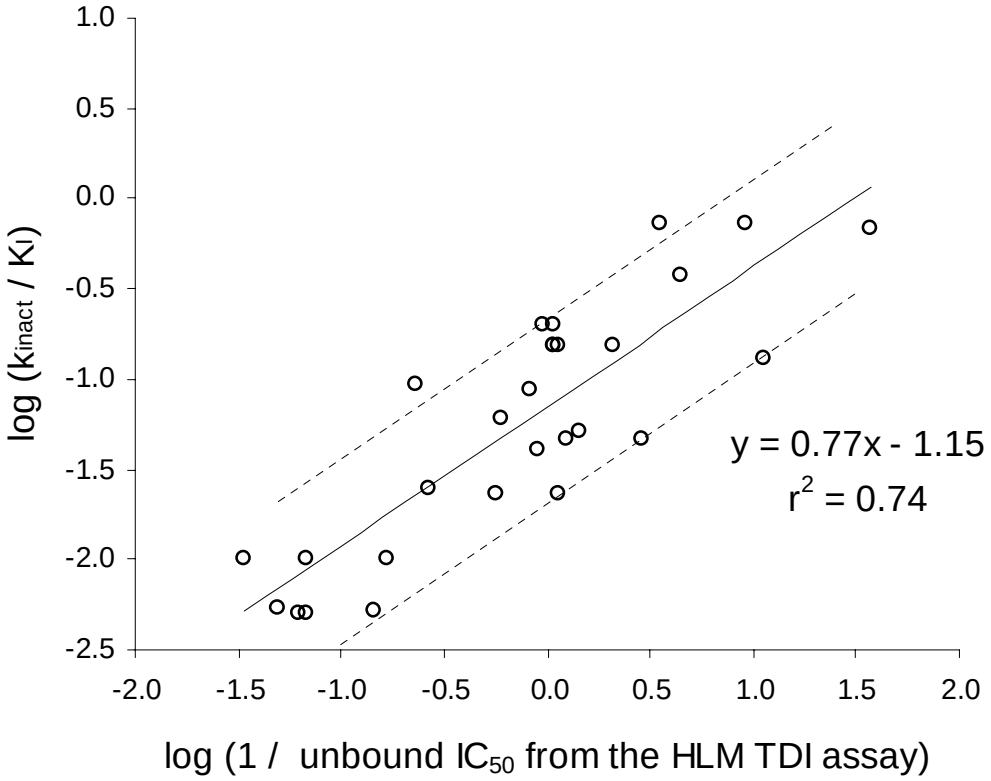
# Figure 4

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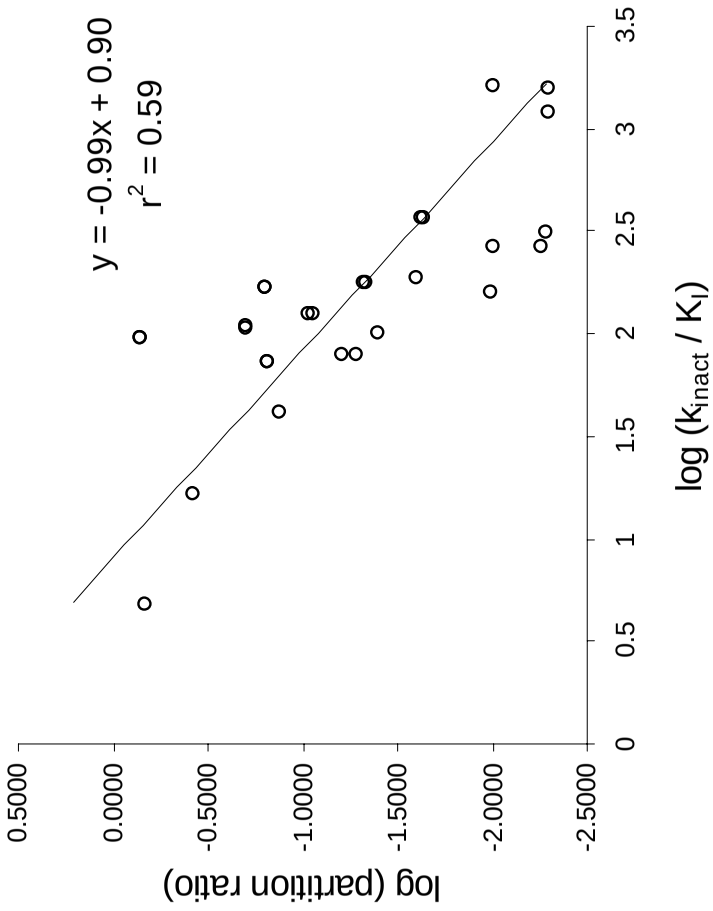
# Figure 5

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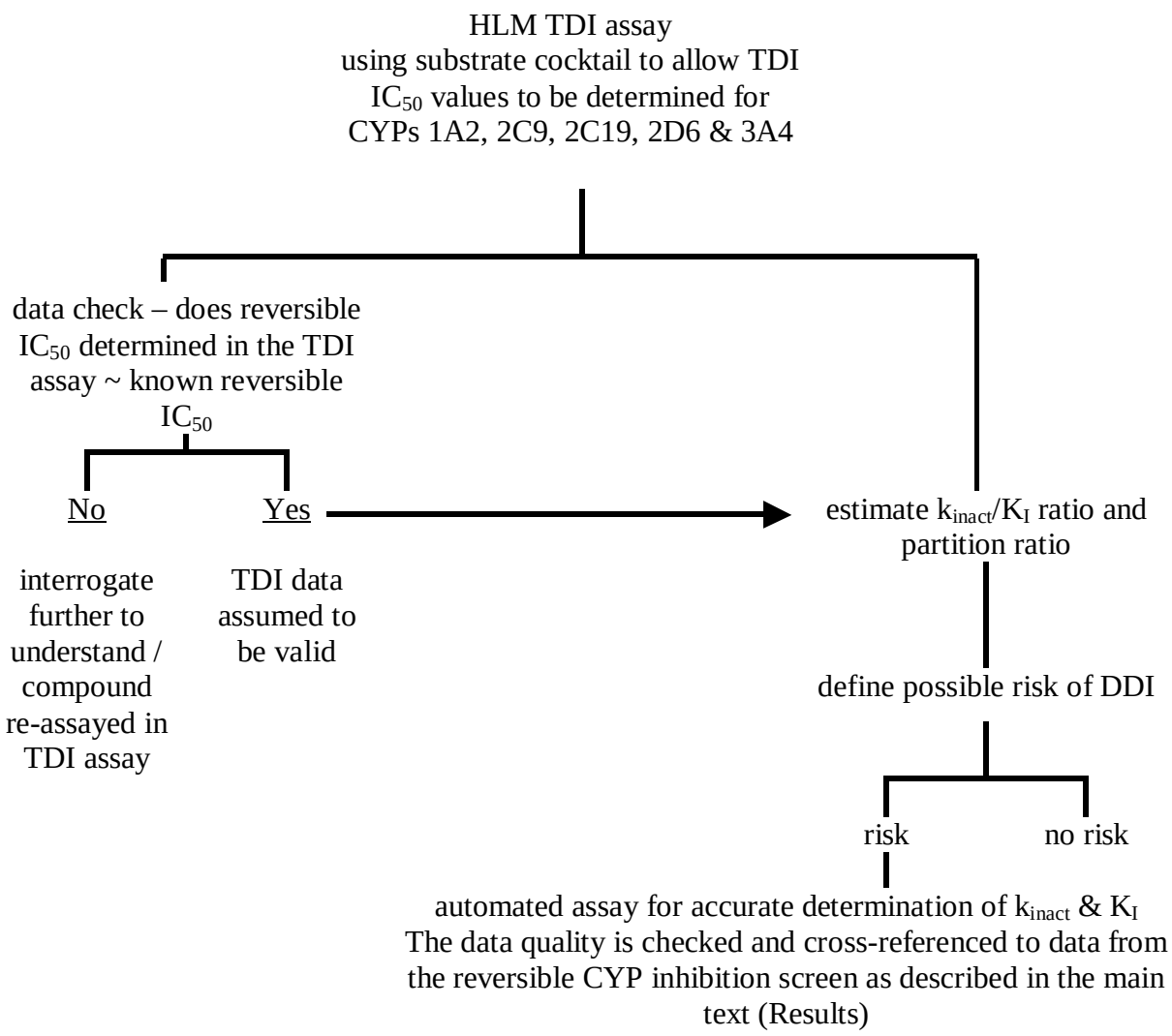
# Figure 6

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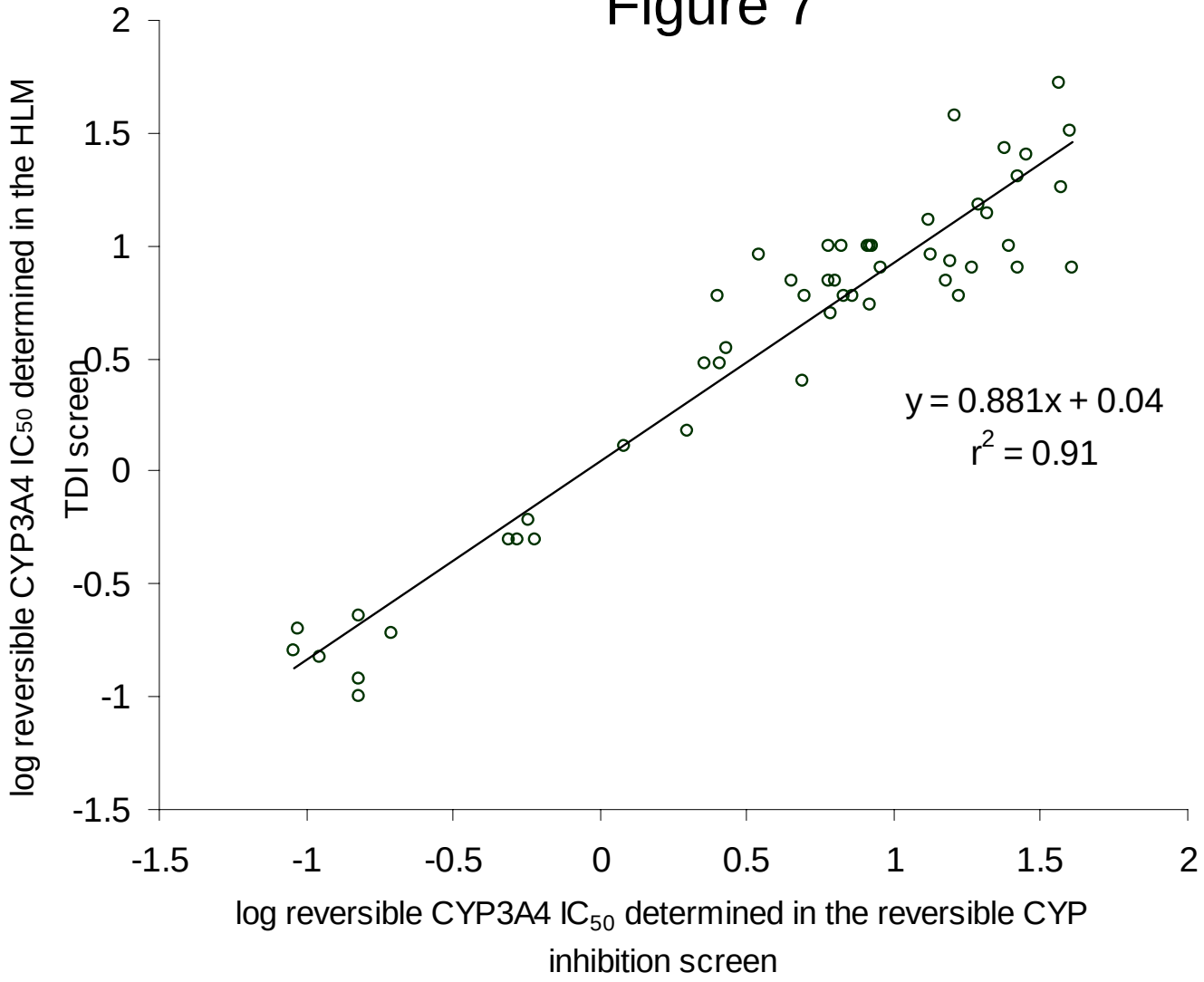
# Figure 7

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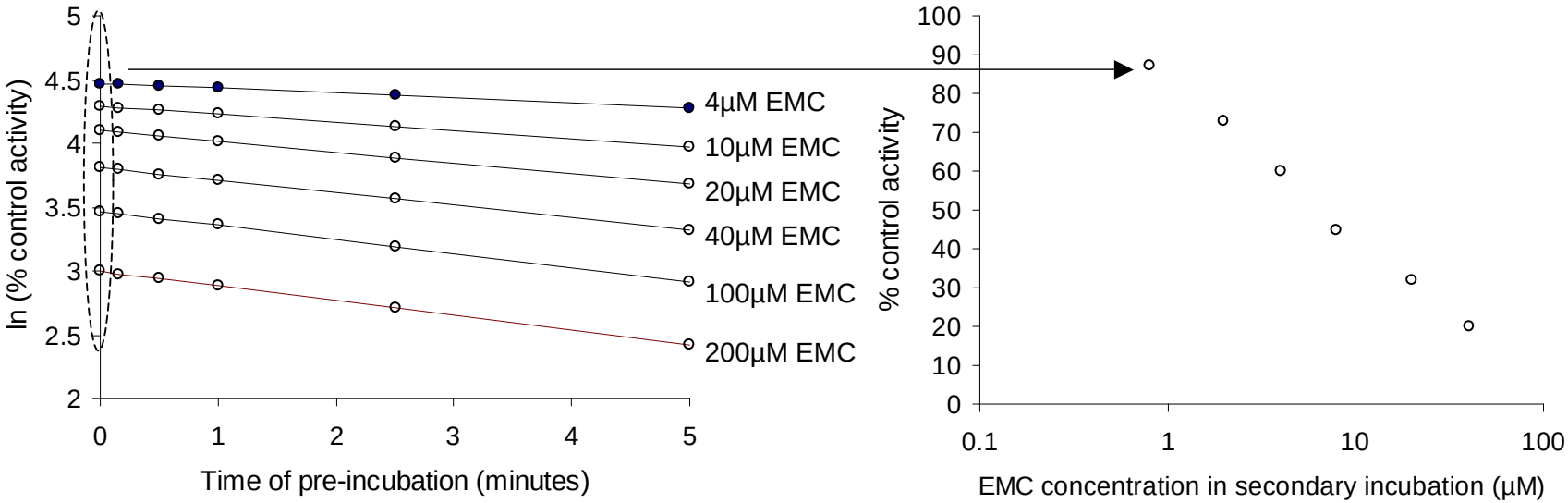
# Figure 8

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