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Evaluation Of Single Point And IC_{50} Shift Assays For Measuring Time-Dependent Inhibition Of Drug Discovery Compounds

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Abstract

Assessment and early detection of new chemical entities (NCEs) which inhibit cytochrome (CYP) P450 enzymes is of great importance in reducing the development of compounds which can cause drug-drug interactions (DDIs). In particular, irreversible time dependent inhibition (TDI) is of concern for its inhibition results in inactivated enzyme which must be re-synthesised in order to regain activity. Therefore such compounds which cause TDI have greater potential to cause longer lasting DDIs. Unlike reversible inhibition where there are guidelines from the FDA[1], current opinion on investigating TDI is quite varied with little detailed recommendations. At present there is divided opinion on experimental methods and interpretation of data, as explored by the PhRMA review (2009)[2].
The aim of this study is to evaluate different assay designs, and subsequent data analysis methodology for measuring the extent of TDI for known time-dependent inhibitors. In particular we have focused on CYP3A4 activity using midazolam as the probe substrate, analyzing 1'-hydroxymidazolam formation by LC-MS/MS. Firstly, we look to explore loss of activity with a single concentration of inhibitor, comparing the percentage inhibition measured with 30 minute pre-incubations with/without NADPH and correcting for vehicle activity. By comparing different equations[3,4] we will analyse the impact of reversible inhibition on interpretation and accuracy of TDI data. The study then looks to correlate this single point data with IC_{50} shift data, investigating the impact of using a dilution step. Finally, we aim to evaluate the predictive qualities of single point TDI studies and IC_{50} shift assays on estimating potential *in vivo* DDIs. By assessing both of these initial drug discovery phase TDI screens we propose a reversible inhibition and TDI screening platform to cover early phase compounds, which should enable early accurate decisions to be made regarding development of those compounds which could potentially cause DDIs.

Introduction

Assessing the potential of a compound to inhibit a specific cytochrome P450 enzyme is important as co-administration of compounds may result in one or both inhibiting the other's metabolism.

Experimental analysis of test compounds causing TDI occurs by pre-incubating human liver microsomes for 30 minutes with inhibitors, with/without NADPH followed by a specific substrate incubation where the amount of TDI can be analysed by the decrease in formation of metabolite.

Assay conditions can vary from lab to lab considerably, dependent upon whether steps are taken to minimise reversible inhibition.

General variations include addition of a 10x dilution step[8] before the substrate incubation (resulting in pre-incubation at a higher protein concentration), increasing the substrate concentration to 5-10x K_m and having a much longer 'pre-incubation' time opposed to 'incubation' time.

We aim to discuss the effect of these different assay conditions on data interpretation of TDI and utilisation of the different assays for a high throughput screening program.

Methods

Test compounds: The following known time dependent inhibitors were selected as our test set for this investigation. The concentrations stated were used in the single point assay and as the top concentration for the IC_{50} shift experiments. Mibefradil (2µM), Mifepristone (50µM), Verapamil (50µM), Diltiazem (50µM), Troleandomycin (25µM), Erythromycin (50µM), Clarithromycin (25µM), Azamulin (2µM), Ritonavir (2µM).

Single point percent inhibition: Test compounds were incubated with human liver microsomes (HLM) (1mg/mL protein) in the presence and absence of NADPH (1mM) for 30 min pre incubation. Samples were diluted 1:10 in 0.1M phosphate buffer containing 1mM NADPH and 12.5µM Midazolam (5x Km) and incubated for 5 min, after which an aliquot was taken and terminated into methanol containing internal standard. Incubations in the absence of test compound were also performed. Samples were centrifuged for 30 min at 4ºC, supernatant removed and diluted 1:2 into water containing 0.1% formic acid in preparation for analysis.

IC_{50} Shift determination: Test compounds were incubated at 7 different concentrations with HLM (0.1mg/mL protein) in the presence and absence of NADPH (1mM) for 30 min pre incubation (0.25% DMSO). Substrate was added along with further NADPH (2.5µM Midazolam (K_m), 1mM NADPH) and the reaction incubated for 5 min. Test compound was also incubated at 7 concentrations for the 5 min substrate incubation only with no pre incubation. Aliquots were terminated into methanol containing internal standard. Samples were centrifuged and prepared for analysis as above. For those assays that involved a dilution step: compounds were incubated with HLM (1mg/mL protein) and were diluted 1:10 after the 30 min pre incubation. NADPH substrate concentrations remained the same with substrate concentration at either 2.5µM (K_m) or 12.5µM (5xK_m).

Analytical methods: Samples were analyzed by LC-MS/MS using standard Cyprotex methods, monitoring for the metabolite, 1'-hydroxymidazolam.

Data processing: In all experiments the response is equal to peak area of metabolite/ peak area of internal standard. For the single point inhibition the percent inhibition was calculated using either Equation 1 or 2. For the IC_{50} determination the percentage of control is equal to response at XµM/ Response at 0µM x 100%. Standard curve fitting algorithms used to determine the IC_{50}:

IC_{50} values were determined for all three conditions – 30 min pre inc plus NADPH, 30 min pre inc in minus NADPH and 0 min pre inc. The fold shift was determined by; SHIFT = IC_{50} NADPH ^{MINUS} / IC50 NADPH ^{PLUS}

Results

Single Point Inhibition

(Cloe Screen Time-dependent Inhibition assay)

The PhRMA Perspective paper on TDI[2] suggests '% activity loss' in a single point TDI assay should be calculated using equation 1 below. However this does not account for any reversible inhibition which may occur. Equation 2 accounts more fully for total inhibition with a 30 minute pre-incubation.

Equation 1 (Obach *et al*, 2007[4])

$$\text{Percent activity loss} = \left(100 * \left(\left(\frac{R_{\text{Test cmpd - NADPH}}}{R_{\text{Vehicle - NADPH}}}\right) - \left(\frac{R_{\text{Test cmpd + NADPH}}}{R_{\text{Vehicle + NADPH}}}\right)\right)\right)$$

Equation 2 (Atkinson *et al*, 2005[3])

$$\text{Percent inhibition} = 100 * \left(1 - \left(\left(\frac{R_{\text{Test cmpd + NADPH}}}{R_{\text{Vehicle + NADPH}}}\right) / \left(\frac{R_{\text{Test cmpd - NADPH}}}{R_{\text{Vehicle - NADPH}}}\right)\right)\right)$$

Figure 1 highlights the difference between the two equations for our test set of inhibitors. The percent inhibition varies considerably depending on the equation used. Compounds which are most affected are ritonavir and mifepristone, which if based solely on Equation 1 may have been missed as being potent inhibitors, with percent inhibition values of 3.6% and 42.3% respectively, compared to 72.3% and 80.3% inhibition with Equation 2.

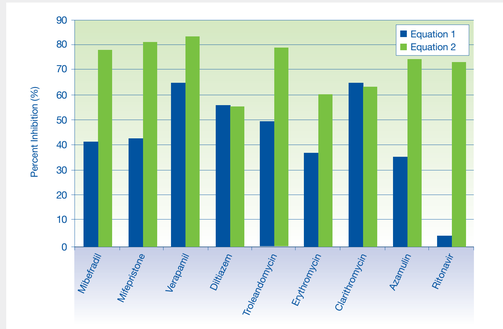


Figure 1 – Percent inhibition data from Cloe Screen time dependent inhibition single concentration assay, calculated using both equations 1 and 2.

Methodology for IC_{50} shift experiments

For investigating the effect of a dilution step on TDI data, four compounds were chosen which span a large potency range, exhibiting direct and time dependent inhibition.

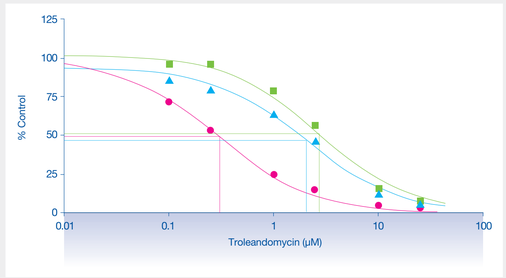
The table below shows how the shifted IC_{50} and the fold shift is affected by a 1:10 dilution.

Compound		Dilution method at K_m	No dilution at K_m	Dilution method at 5x K_m	Comments
Mibefradil	Shifted IC_{50} (µM)	0.33	0.035	0.43	Potent reversible inhibitor. IC_{50} overestimated by dilution factor for both minus and plus NADPH incubations. Fold shift not effected.
	Fold shift	5	4	7	
Mifepristone	Shifted IC_{50} (µM)	0.81	0.41	1.04	Dilution method removed reversible inhibition. Results increase in IC_{50} $NADPH^{MINUS}$. Fold shift increased.
	Fold shift	50	10	36	
Verapamil	Shifted IC_{50} (µM)	1.15	1.07	1.4	Dilution method removes reversible inhibition. Fold shift not determined.
	Fold shift	ND	9	ND	
Diltiazem	Shifted IC_{50} (µM)	3.1	4.82	14	Dilution method removes reversible inhibition. Fold shift not determined.
	Fold shift	ND	8	ND	

Table 1 – A comparison of the effects of using the dilution method on fold shift and shifted IC_{50} for our test set of inhibitors

The results show that adding a dilution affects compounds differently depending on the type of inhibition that occurs. The dilution step also introduces problems into the data analysis. Firstly, a marked difference appears between processing IC_{50} results with initial inhibitor concentration (pre-dilution) opposed to final inhibitor concentration (post-dilution).[7] Pre-incubating at a 10x higher protein concentration can also introduce the possibility of non-specific binding which can decrease the amount of inhibition observed.

From these results the Cloe Screen Cytochrome P450 IC_{50} Shift assay protocol was designed without a dilution step, running the substrate incubation at K_m. Figure 2 shows a typical plot obtained from the assay.



	Predicted IC_{50} (µM)	SE IC_{50}	% Inhibition
0 Minute	2.003	0.318	95.08
30 Minute Minus	2.766	0.385	92.97
30 Minute Plus	0.298	0.025	97.91

Figure 2: Inhibition profile for troleandomycin run through standard conditions for the IC_{50} shift assay, without a dilution step, measuring percent control as a function of inhibition of midazolam 1'-hydroxylation compared to 0µM control.

Correlating single point inhibition data with IC_{50} shift data

Good correlation was observed upon ranking the test set of compounds on percent inhibition and shifted IC_{50}. Azamulin and ritonavir were 2 compounds which fell slightly out of line due to having a marked reversible inhibition element which is underestimated in the single point TDI assay.

In agreement with literature[4,6] we did not observe any correlation with shifted IC_{50} or percent inhibition and fold shift.

Using single point and IC_{50} shift data for DDI prediction

According to Atkinson *et al.*[3], percent inhibition values can be used to project a 'shifted IC_{50}'. However the values we obtained from using the equation did not correlate with our experimentally determined shifted IC_{50} values. This is likely to be attributable to the different methods used by each assay.

The k_{inact}/K_I ratio can be used as an indicator of DDI risk in early screening and can be calculated from IC_{50} data using the following equation.

Equation 3: (Maurer et al 2000[5])

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

The equation has been shown to predict very well and our calculated k_{inact}/K_I correlates well with experimental literature values for our test set. A plot of rank by our data vs. literature data is shown in Figure 3.

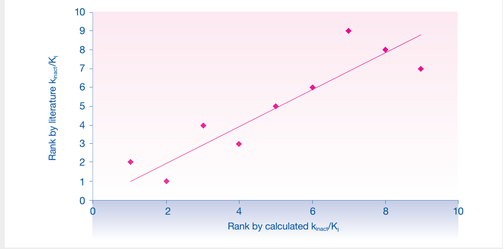


Figure 3: An assessment of correlation between our calculated k_{inact}/K_I values using the shifted IC_{50} data and equation 3, with experimentally determined literature values for k_{inact}/K_I.

Predicting drug-drug interactions from IC_{50} data

Many models and equations have been developed to transform in vitro P450 inhibition data into meaningful *in vivo* predictions. However these equations are complex and require additional compound information from later stage studies and *in vivo* data. Data from k_{inact}/K_I studies is shown to predict DDI well although very dependent upon input from accurate [I].[4] Models which instead use shifted IC_{50} values due to the ease of obtaining data compared to a more in depth k_{inact}/K_I studies, have been shown to often under predict the DDI, with a high percentage of false negative results.[6]

We propose a P450 inhibition screening decision tree which can be used to filter discovery compounds through several inhibition assays reducing numbers and gaining more insight at each level. This is with a view to obtaining the most information only on important compounds from which accurate predictions on DDI and decisions on necessary *in vivo* assays can be made.

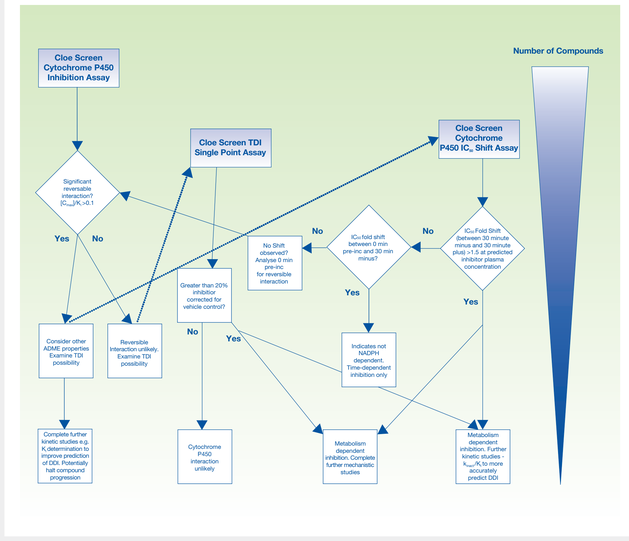


Figure 4: P450 Inhibition Decision Tree

Conclusions

- Decisions on early discovery/development compounds should be made based on the total inhibition (reversible and time-dependent). Our single point, time-dependent inhibition screen is analysed using equation 2 to deliver the most information about compounds screened in a high-throughput manner.
- The experimental conditions for an IC_{50} shift assay should encourage all inhibition to give the most accurate shifted IC_{50} value with a 30 minute pre-incubation. A dilution step should not be added as this can skew data depending on the inhibitor concentration used for calculations or microsomal binding at higher protein concentrations.
- Shifted IC_{50} values from the high throughput Cloe Screen IC_{50} shift assay, correlate well with k_{inact}/K_I values and as such can be used as an indicator of DDI risk and allow decisions to be made for further *in vitro* screens or *in vivo* work.

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