Development of high throughput assays for the screening of reversible and mechanism-based cytochrome P450 inhibition by test compounds

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ABSTRACT

Assessment of cytochrome P450 (CYP450) inhibition is now performed early in drug development to decrease the likelihood of progressing a compound with the potential to cause drug-drug interactions. Screening for reversible CYP450 inhibition, which is widely performed, does not have the ability to detect mechanism-based inhibition of CYP450 by test compounds. The prevalence, and clinical implications, of mechanism-based CYP450 inhibition has placed greater emphasis on the early detection of compounds with this potential. We have developed and validated a high throughput reversible CYP450 inhibition assay using human liver microsomes and industry recommended probe substrates. Five CYP450 isoforms are screened (CYP1A, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) with two substrates utilised for CYP3A4 (midazolam and testosterone). The IC_{50} can then be calculated for the test compound against the five CYP450 isoforms. In parallel we have also developed and validated a preliminary high throughput mechanism-based CYP450 inhibition screen using human liver microsomes and industry recommended probe substrates. A single concentration of test compound is pre-incubated for 30 minutes in the presence and absence of NADPH with corresponding controls. Following pre-incubation an aliquot of this reaction is diluted 10-fold into a sample containing the individual probe substrate and the residual CYP450 enzyme activity assessed. An estimate of the mechanism-based inhibitory potential of the test compounds can then be determined in comparison to the positive controls. We have automated the entire screening process with the use of BasePlate™ liquid-handling robot technology, automated generic fast LC-MS/MS analysis and a custom laboratory-information management system. These assays can be applied to screen a large number of discovery compounds in a cost effective way to identify, and differentiate, those compounds that cause reversible or mechanism-based CYP450 inhibition. This information can be used to make early decisions to avoid the development of those compounds with the potential to cause drug-drug interactions due to CYP450 inhibition.

INTRODUCTION

Metabolic drug-drug interactions following co-administration of drugs may result in either reduced efficacy or increased toxicity. These effects are particularly critical in drugs which have a narrow therapeutic index. Several drugs (e.g. terfenadine, mibefradil, astemizole, cisapride, sorivudine) have either been withdrawn from the market or suffer from restrictions in their prescription for this reason (Tucker *et al*., 2001). The family of cytochrome P450 (CYP450) enzymes are particularly involved in drug-drug interactions as they play a major role in the metabolism of drugs.

Screening for CYP450 inhibition can be expensive and time consuming. For this reason, it is often not considered as an initial filter in the drug discovery process. Cyprotex have developed high quality CYP450 inhibition assays (Cloe®Screen CYP450 Inhibition) which screen for both reversible and mechanism-based CYP450 inhibition. The reversible CYP450 screen utilises a range of test compound concentrations to determine the IC_{50} value. The mechanism-based CYP450 screen uses a single test compound concentration to give a preliminary estimation of whether the test compound acts as a mechanism-based inhibitor. Both screens use industry accepted CYP450 probe substrates rather than fluorescent probe substrates, and human liver microsomes. The use of high capacity automated pipetting system and automated data processing enables rapid screening of compounds in a cost effective manner. By reducing the cost and increasing the speed of the assays, a comprehensive CYP450 inhibition screening package can be performed at an earlier stage in drug discovery thereby reducing the possibility of costly late stage failures.

TABLES

TABLE 1.

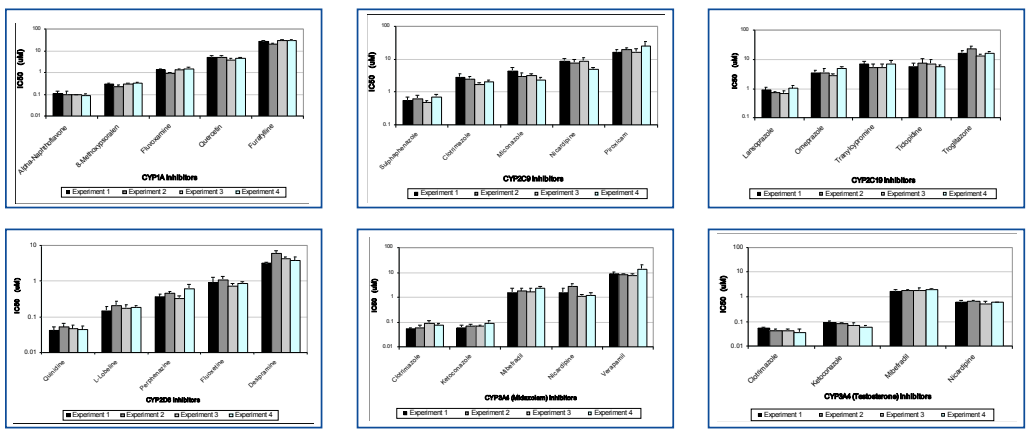
Comparison of in-house IC_{50} Values for Reversible CYP450 inhibition with Literature Values

Inhibitor	Isoform	Cloe® Screen IC_{50} (μM) ± SE	Literature IC_{50} (μM)	Reference
α-Naphthoflavone	CYP1A	0.10 ± 0.01	0.12	Bu *et al*. 2001
Sulphaphenazole	CYP2C9	0.62 ± 0.11	0.5	Back *et al*. 1988
Tranylcypromine	CYP2C19	6.31 ± 0.99	9	Dierks *et al*. 2001
Quinidine	CYP2D6	0.05 ± 0.005	0.02	Moody *et al*. 1999
Ketoconazole	CYP3A4 (midazolam)	0.07 ± 0.01	0.07	Nomeir *et al*. 2001
Ketoconazole	CYP3A4 (testosterone)	0.08 ± 0.006	0.07	Riley *et al*. 1998

FIGURES

FIGURE 1.

Intra and Inter Assay Variability for CYP1A, CYP2C9, CYP2C19, CYP2D6, CYP3A4 (midazolam) and CYP3A4 (testosterone) Reversible Inhibition Assays (data shown mean IC_{50} ± SE n=3)



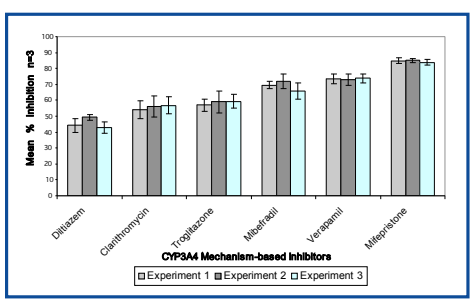


FIGURE 2.

Intra and Inter Assay Variability for CYP3A4 (midazolam) Mechanism-Based CYP450 Inhibition Assay.

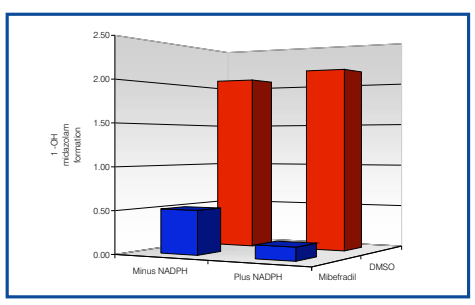


FIGURE 3.

Discrimination between Reversible and Mechanism-Based CYP450 Inhibition by Mibefradil against CYP3A4 (midazolam)

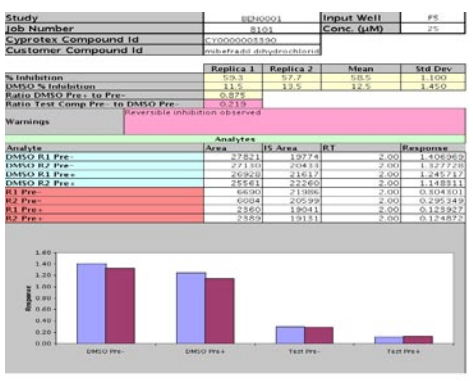


FIGURE 4.

Example Data Processing for Mechanism-Based Inhibition CYP3A4 (midazolam) Assay using Midefradil

METHODS

Reversible CYP450 Assay Conditions

Six enzyme specific reactions are screened separately using human liver microsomes and probe substrates for the five main CYP enzymes (CYP1A, CYP2C9, CYP2C19, CYP2D6, and CYP3A4). Each reaction is performed under linear conditions with respect to time and protein. The substrate concentrations selected are equivalent to the K_m of each reaction. Six test compound concentrations (0.05, 0.25, 0.5, 2.5, 5, 25 μM, final DMSO concentration 0.25 %) are screened in order to generate an IC_{50} value.
Selective known inhibitors are screened alongside the test compounds as positive controls for the experiment.

The six isoform specific reactions and corresponding reversible inhibitors are listed in the table below:

CYP Isoform	Reaction	Reversible Inhibitor
CYP1A	Ethoxyresorufin *O*-dealkylation	α-Naphthoflavone
CYP2C9	Tolbutamide 4-hydroxylation	Sulphaphenazole
CYP2C19	*S*-Mephenytoin 4-hydroxylation	Tranylcypromine
CYP2D6	Dextromethorphan *O*-demethylation	Quinidine
CYP3A4	Midazolam 1-hydroxylation	Ketoconazole
CYP3A4	Testosterone 6β-hydroxylation	Ketoconazole

Mechanism-Based CYP3A4 Assay Conditions

A single concentration of test compound, equivalent to the highest concentration screened in the reversible CYP450 assay (final DMSO concentration 0.25 %), is incubated with human liver microsomes (at 10 x concentration present in reversible assay) in the presence and absence of NADPH for 30 minutes. Corresponding control incubations are performed in the absence of test compound. After 30 minutes an aliquot of the reaction mixture is diluted 10-fold into buffer containing midazolam, at a concentration equivalent to 5 K_m, and additional NADPH. Selective known mechanism-based inhibitors are screened alongside the test compounds as positive controls for the experiment. A % inhibition value for the test compound is then determined following pre-incubation in the presence of NADPH for the single concentration tested.

Sample Processing and Analysis

All reactions are terminated by the addition of methanol. Ethoxyresorufin O-dealkylation is monitored by fluorescence using a Tecan Spectrofluor Plus plate reader (excitation wavelength = 535nm, emission wavelength = 595nm). The remaining isoform reactions are analysed by LC-MS/MS. For these reactions, the incubation plates are centrifuged to precipitate the microsomal protein (2500rpm for 30min at 4°C), the supernatants for the individual isoform reactions are combined, and the cassettes are monitored for the five isoform-specific metabolites by LC-MS/MS. Internal standard is included in all samples.

We have automated the entire processes so that we can analyse up to 90 test compounds at one time plus controls, with the use of a BasePlate™ liquid handling technology, cassette LC-MS/MS analysis and a tailored laboratory-information management system - enabling the analysis of large numbers of discovery compounds in a cost effective manner. To enable automation the liquid handling methods are based upon 10 mM DMSO stock solutions of test compounds as many companies now routinely store their compounds in DMSO. In addition, it is important to use a solvent which solubilises the majority of compounds and is not subject to evaporation; DMSO tends to be the best solvent for this purpose.

RESULTS

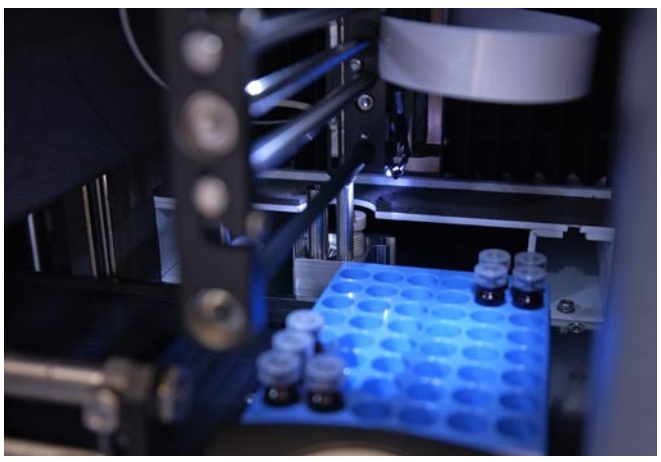
Reversible CYP450 Inhibition Assay

Following the determination of time/protein linearity and the K_m for each of the CYP450 isoform specific metabolites the intra- and inter-assay variability was determined for each reaction. Figure 1 shows the variability for each specific reaction with at least 4 specific inhibitors screened for each isoform. The IC_{50} values obtained showed a high level of consistency between, and within, each assay run.

The following compounds were then selected for use as positive controls in the reversible CYP450 inhibition assay: CYP1A α-naphthoflavone, CYP2C9 sulphaphenazole, CYP2C19 tranylcypromine, CYP2D6 quinidine and CYP3A4 ketoconazole. Table 1 details the IC_{50} values obtained for these compounds and the comparison to literature IC_{50} values. Considering IC_{50} values are inherently variable between assay systems the IC_{50} values obtained in-house were highly comparable to the literature values.

Mechanism-Based CYP3A4 Inhibition Assay

The validation of the CYP450 mechanism-based assay is currently ongoing therefore the data on CYP3A4 with midazolam as the substrate is presented here. A literature search was performed to identify a selection of compounds, of varying potency, which are mechanism-based inhibitors of CYP3A4. Six compounds (diltiazem, clarithromycin, troglitazone, mibefradil, verapamil and mifepristone) were selected and the intra- and inter-assay variability determined which is shown in Figure 2. The results show that there is a high level of consistency between, and within, each assay run over a range of inhibition.



Mibefradil is both a reversible and mechanism-based CYP3A4 inhibitor. The results from the mechanism-based screen show that it is possible to both detect and discriminate between, the reversible CYP450 inhibition and the mechanism-based CYP450 inhibition associated with this test compound (Figure 3).

A screenshot of the custom in-house automated data processing system that allows for the rapid evaluation of a test compounds inhibitory potential is shown in Figure 4.

CONCLUSIONS

It is well recognised within the pharmaceutical industry that being able to identify safe, efficacious compounds with favourable pharmacokinetic properties early in the drug discovery process will save valuable time and money. Early stage identification of a compounds potential to inhibit CYP450 enzymes is an essential component of drug development to minimise any potential drug-drug interactions. Reversible CYP450 inhibition is now routinely screened, however, there is an increasing requirement and demand to understand the potential of a compound to act as a mechanism-based CYP450 inhibitor. We have developed high quality, rapid, cost effective screens using industry approved probe substrates to understand the inhibitory potential of a test compound against CYP450. The data obtained from these screens can be used for directing chemistry and identifying potential leads, as well as for building databases to improve predictive models.

The Cloe®Screen CYP450 inhibition screen offers high throughput, reproducible assays. For reversible inhibition we generate IC_{50} values which are comparable with literature values for known inhibitors. We also generate a preliminary single point inhibition value which can be used to estimate the potential of a compound to act as a mechanism-based inhibitor for CYP3A4 (midazolam). Further CYP450 isoforms (CYP1A, CYP2C9, CYP2C19, CYD2D6 and CYP3A4 [testosterone]) are currently being validated in the mechanism-based inhibition assay.

Once a compounds' inhibitory potential has been evaluated further studies can be performed to characterise the inhibition. For reversible inhibition K_i values for the test compound can be determined and for mechanism-based inhibition K_i and K_{inact} can be determined. These values can then be used to estimate any potential *in vivo* drug-drug interactions for the test compound.

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