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Comparison of cytochrome P450 inhibition assays for drug discovery using human liver microsomes with LC–MS, rhCYP450 isozymes with fluorescence, and double cocktail with LC–MS

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Abstract

The disparity of IC_{50}s from CYP450 inhibition assays used to assess drug–drug interaction potential was investigated, in order to have evidence for selecting a reliable in vitro CYP450 inhibition assay to support drug discovery. Three assays were studied: individual rhCYP isozymes and corresponding coumarin derivative-probe substrates with fluorescent detection, human liver microsomes (HLM) and cocktail drug-probe substrates with LC–MS detection, and double cocktail rhCYP isozymes mix and drug-probe mix with LC–MS detection. Data comparisons showed that the rhCYP-fluorescent assay and the cocktail assay with HLM–LC–MS had weak correlation. Detection method and probe substrates were shown to not be the major cause of the disparity in IC_{50}s. However, the enzyme source and composition (HLM versus, rhCYP) caused disparity in IC_{50}s. Specifically, the high concentrations of CYP isozymes often used with HLM-based assays produced high probe substrate conversion and test compound metabolism, which should both contribute to artificially higher IC_{50}s. Non-specific binding of substrate to higher concentration proteins and lipids in the HLM-based assays should also contribute to higher IC_{50}s. The modified double cocktail assay was found to overcome limitations of the other two assays. It uses an rhCYP isozymes mix, drug-probe substrate mix, low protein concentration, and LC–MS detection. The double cocktail assay is sensitive, selective, and high throughout for use in drug discovery to provide an early alert to potential toxicity with regard to drug–drug interaction, prioritize chemical series, and guide structural modification to circumvent CYP450 inhibition.

Keywords: CYP450 inhibition; Drug–drug interaction (DDI); Metabolism; Fluorescent; LC–MS; High throughput; Double cocktail assay

1. Introduction

One of the challenges of drug discovery is to predict drug–drug interaction mediated by CYP450 inhibition that can reduce drug clearance and lead to toxicity (Zlokarnik et al., 2005). Several drugs (e.g., Posicor, Seldane, Hismanal) have been withdrawn from the market due to drug–drug interactions (Rostami-Hodjegan and Tucker, 2004). Measurement of CYP450 inhibition in drug discovery can provide data for: an early alert to CYP450 inhibition potential, development of structure–CYP450 inhibition relationships that can be used to overcome inhibition by structural modification, and selection of lead compounds having a good profile. Therefore, many pharmaceutical companies have implemented high throughput CYP450 inhibition assays along with other ADME/TOX assays to enhance the quality of development candidates and reduce attrition (Rodrigues and Lin, 2001; Riley and Grime, 2004; Jenkins et al., 2004; Saunders, 2004; Kassel, 2004; Kerns and Di, 2003; Li, 2001; Di and Kerns, 2003).

In vitro CYP450 inhibition studies are also of great importance for clinical trials for two main reasons (Obach et al., 2005, 2006; Huang et al., 1999). First, in vitro CYP450 inhibition data showing negligible inhibition can be used as the sole basis to conclude that the compound lacks drug–drug interaction

Abbreviations: ADME/TOX, absorption, distribution, metabolism and excretion/toxicity; AMMC, 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; CYP450, cytochrome P450; HLM, human liver microsomes; LC–MS, liquid chromatography and mass-spectrometry; MFC, 7-methoxy-4-trifluoromethylcoumarin; MRM, multiple reaction monitoring; PhRMA, pharmaceutical research and manufacturers of America; SAR, structure–activity relationship

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potential and requires no clinical drug-drug interaction studies. Second, positive in vitro CYP450 inhibition data can be used to plan clinical study strategies and to minimize the number of studies required. PhRMA has provided general guidelines on conducting in vitro and in vivo drug–drug interaction studies (Bjornsson et al., 2003), and leaders in the field are working towards standardized and validated assays (Walsky and Obach, 2004). Approaches have been developed to predict in vivo drug-drug interaction using in vitro data (Shou, 2005). Therefore, high quality in vitro data are crucial for drug discovery programs in determining the necessity and/or design of clinical trials.

Various in vitro assays for CYP450 inhibition have been developed and adapted for drug discovery. They differ in CYP enzyme source and composition (i.e., recombinant [cDNA expressed] human CYP [rhCYP] isozymes, human liver microsomes [HLM]), probe substrates (i.e., drugs, coumarin derivatives), and detection (i.e., LC–MS, radioactivity, fluorescence, luminescence) (Zlokarnik et al., 2005). The two most commonly used assays are: (a) rhCYP isozymes with coumarin derivative probe substrates and fluorescence detection (rhCYP-fluorescent)[1] (Crespi et al., 1997, 1998), and (b) HLM with drug probe substrates and LC–MS detection (HLM–LC–MS) (Dierks et al., 2001). The rhCYP-fluorescent assay is high throughput (100–1000 compounds/day) and is often used at the early stages of drug discovery to assess potential CYP450 inhibition liabilities of thousands of compounds. Because the fluorescent-probe substrates are not specific for each CYP isozyme, a single purified rhCYP enzyme is used in each assay and HLM are not used. The HLM–LC–MS assay is medium throughput (10–100 compounds/day) and it is typically used at late stages of drug discovery and development. Because the drug-probe substrates used with the HLM–LC–MS assay are specific for each CYP enzyme, different mixed isozyme sources, such as HLM, can be used. To increase throughput, a cocktail of specific drug-probe substrates is used with HLM and the signal due to each substrate metabolite is independently monitored using the specificity of LC–MS. This approach increases throughput by determining inhibition of several isozymes simultaneously. If an organization is using both assays at different stages of drug discovery and development, data consistency is crucial in order to prioritize compounds, guide synthetic modification and maintain confidence from data end-users, such as medicinal chemists. Studies have been conducted to compare results obtained from the assays (Zlokarnik et al., 2005; Favreau et al., 1999; Bapiro et al., 2001; Nomeir et al., 2001; Cohen et al., 2003; Weaver et al., 2003). Some studies showed good correlations and some showed weak correlations, depending on the assay conditions used, specific CYP450 isozymes studied and test compounds (inhibitors) included in the studies. There has been speculation but further evidence for the cause of these data disparities is warranted.

In order to have evidence for selection of a reliable CYP450 inhibition assay to support drug discovery lead selection and optimization, we investigated the data differences between methods and the procedural differences that lead to these disparities. Three assays were studied: (a) rhCYP-fluorescent, (b) HLM–LC–MS, and (c) double cocktail. The double cocktail assay is a variation of an assay by Weaver et al. (2003) in which a mixture (cocktail) of specific drug-probe substrates is used with a cocktail of rhCYP isozymes. The double cocktail assay offers flexibility for selection of CYP isozyme concentrations in optimizing the enzyme kinetics. It also offers advantages compared to limitations of the rhCYP-fluorescent and HLM–LC–MS methods.

2. Experimental

2.1. Materials

All reagents used were of the highest grade commercially available. The test compounds were obtained from Aldrich and Sigma Chemical Co. (St. Louis, MO), Fluka (Ronkonkoma, NY), and Wyeth compounds were obtained from Wyeth Research (Princeton, NJ). NADPH regenerating agent Solution A and Solution B (NADPH-RS), recombinant human (cDNA-expressed) CYP3A4, 2D6 and 2C9 Supersomes™, fluorescent substrates (7-benzyloxy-4-(trifluoromethyl)-coumarin [BFC], 3-[2 -(*N*, *N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin [AMMC], and 7-methoxy-4-(trifluoromethyl)-coumarin [MFC]) and drug-probe substrates (midazolam, bufuralol and diclofenac), and metabolite standards (1′-hydroxymidazolam, 1′-hydroxybufuralol and 4′-hydroxydiclofenac) were obtained from BD Gentest (Woburn, MA). Human liver microsomes (mixed gender, pool of 50) were purchased from XenoTech (Lenexa, KS). Ninety-six well plates were obtained from Corning Incorporated (Acton, MA).

2.2. Instrumentation and software

A Packard Multiprobe™ II EX HT (eight probe) robot with WinPrep™ software was applied for sample preparation (Perkin-Elmer, Downers Grove, IL). A Jitterbug microplate incubator shaker (Boekel Scientific, Bridgeport, NJ) was used for maintaining reactions at 37 °C. Centrifugation used an Eppendorf centrifuge 5810R (VWR, Rochester, NY). Model 1100 HPLC pumps (Agilent Technology, Piscataway, NJ) were used for HPLC separations. A CTC Twin Pal autosampler (LEAP Technologies, Carrboro, NC) equipped with six cooled well plate holder drawers (12 tray capacity), a syringe injection valve, and a 10-port valve, to which 2 trapping cartridges (Keystone Aquasil C18 10 mm × 2.1 mm, 5 μm, Bellefonte, PA) were attached, was utilized. An analytical column was applied after the trapping cartridges (Keystone Aquasil C18 50 mm × 2.1 mm, 5 μm, Bellefonte, PA). A triple quadrupole Micromass Quattro Micro™ mass spectrometer (Waters, Milford, MA), with electrosprary ionization (ESI) was employed for sample analysis. Instruments were controlled by Masslynx software (Version 4.0, Waters, Milford, MA). A FLUOstar™ fluorescent plate reader was applied for quantitation in the fluorescent method (BMG LABTECH Inc., Durham, NC)

[1] http://www.gentest.com/.

2.3. HPLC conditions

Column: Aquasil C18, 50 mm × 2.1 mm, 5 μm (Thermo, Bellefonte, PA).
Loading: 0.1% formic acid in water, flow rate 3 mL/min.
Mobile phase: $A = 0.1\%$ formic acid in water, $B = 0.1\%$ formic acid in acetonitrile.

Time	%A	%B
Gradient		
0	100	0
1.9	5	95
2.0	100	0
3.0	100	0

Flow rate: 0.9 mL/min, split 0.2 mL/min to MS.
Detection: ESI+, MRM.
Injection volume: 20 μL.
Column temperature: ambient.

2.4. rhCYP-fluorescent CYP450 inhibition assay

This assay used individual fluorescent probe substrates with individual rhCYP isozymes and fluorescent detection according to the published method (Crespi et al., 1997; Crespi et al., 1998; see footnote 1). The probe substrates used for each isozyme were: BFC for CYP3A4, AMMC for CYP2D6 and MFC for 2C9. Enzyme and substrate concentrations are shown in Table 1. The substrate concentrations were at the K_m for the assay. The final DMSO concentration was 0.2%. Test compounds (inhibitors) were assayed in duplicate. The percent inhibition was determined at 3 μM test compound concentration. The IC_{50} was determined using a six-point concentration curve with three-fold serial dilution. Depending on the starting (highest) concentration, the concentrations for each compound were different. IC_{50} was calculated using the reported method (see footnote 1).

2.5. HLM–LC–MS CYP450 inhibition assay

This assay used a cocktail of drug-probe substrates that are specific for each isozyme with HLM and LC–MS detection, according to the published method (Dierks et al., 2001). Each test compound was dissolved in DMSO at a concentration 500 times higher than the final assay concentration. Ten microliters of each DMSO stock solution were added by the robotic system to 990 μL of 0.1 M phosphate buffer at pH 7.4. Each solution was mixed and 50 μL of each diluted test compound was added to a well of a 1 mL 96-well polypropylene plate. Fifty microliters of cofactor with NADPH regenerating system (NADPH-RS) were added to each well. The plate was incubated at 37 °C for 10 min. Enzyme-substrate mix was prepared by pre-warming the 0.1 M phosphate buffer at 37 °C for at least 10 min and the HLM and substrates were added right before addition to the reaction plate. Enzyme-substrate mix (100 μL) was added to each well to start the reaction. For human liver microsomes, final protein concentrations of 0.5 and 0.1 mg/mL were both tested. The drug-probe substrate concentrations and estimated individual CYP enzyme concentrations from human liver microsomes are shown in Table 1. After a 20-min incubation at 37 °C in the Jitterbug incubator, the reaction was stopped by addition of 150 μL of cold methanol to denature the protein. Methanol was used instead of acetonitrile to enhance HPLC peak shapes. The solution was centrifuged at 10 °C for 10 min at 3000 rpm and the supernatant was transferred for LC–MS–MS analysis. The final assay conditions were: 1.3 mM NADPH, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U/mL glucose-6-phosphate dehydrogenase, 0.2% DMSO, and 0.5 or 0.1 mg/mL protein concentration of HLM. Compounds were tested in duplicate. The concentrations of the metabolites were determined from standard curves using commercially available metabolite standards. Internal standard was not used in the assay, because there is no ideal internal standard that has structural similarities to all three of the metabolites monitored. Instead, the full-scale solution (no inhibitor compounds added) was injected before and after every six injections as the 100% signal and the area counts of the two standard runs (before and after) were averaged to obtain

Table 1
Comparison of CYP450 inhibition assay conditions

	Methods			
	rhCYP-fluorescent	HLM–LC–MS[a]		Double cocktail
CYP enzymes	rhCYP isozymes	HLM, 0.5 mg/mL	HLM, 0.1 mg/mL	Mixed rhCYP isozymes
CYP3A4	5.0 pmol/mL	42 pmol/mL	8.4 pmol/mL	0.78 pmol/mL
CYP2D6	7.5 pmol/mL	4.2 pmol/mL	0.84 pmol/mL	0.28 pmol/mL
CYP2C9	20 pmol/mL	37 pmol/mL	7.4 pmol/mL	0.55 pmol/mL
Substrate	3A4-BFC (50 μM) 2D6-AMMC (1.5 μM) 2C9-MFC (75 μM)	3A4-midazolam (2.5 μM) 2D6-bufuralol (5 μM) 2C9-diclofenac (10 μM)		3A4-midazolam (2.5 μM) 2D6-bufuralol (5 μM) 2C9-diclofenac (10 μM)
Cofactor	NADPH-RS	NADPH-RS		NADPH-RS
Incubation time	30–45 min	20 min		20 min
Detection	Fluorescent	LC–MS–MS		LC–MS–MS

[a] Calculated based on the total CYP450 content of 415 pmol/mg (Xenotech, LLC. Lot # 0510139) and distribution of each isozymes (Rodrigues, 1999) (3A4 = 20%, 2D6 = 2.0% and 2C9 = 18%).

Table 2
LC–MS–MS MRM conditions used for HLM–LC–MS and double cocktail assays

Isozymes	Substrate	Metabolite	MRM (m/z)[a]	Cone voltage (V)	Collision energy (eV)
CYP3A4	Midazolam	1′-Hydroxymidazolam	342 > 203	25	40
CYP2D6	Bufuralol	1′-Hydroxybufuralol	278 > 186	40	22
CYP2C9	Diclofenac	4′-Hydroxydiclofenac	312 > 230	25	16

[a] Dwell time 0.2 s and 0.02 interscan delay.

the 100% signal for calculation of % inhibition. The IC_{50} was determined using the 6-point concentration curve. The IC_{50} was derived using the reported method (see footnote 1). The percent conversion was calculated by dividing the post-incubation molar concentration of the probe substrate metabolite by the pre-incubation molar concentration of the probe substrate and multiplying 100.

LC–MS conditions are shown in Instrumentation and Software and Table 2. For 1′-hydroxymidazolam, the commonly used transition (m/z 342 > 324, loss of water) was not used due to non-specificity and a different transition (m/z 342 > 203) was used.

2.6. Double cocktail CYP450 inhibition assay

This assay used a cocktail of drug-probe substrates that are specific for each isozyme with a cocktail of rhCYP isozymes. The method was a modification of a previously published method (Weaver et al., 2003). The final concentration of each substrate and isozyme are shown in Tables 1 and 2. Other procedural steps and conditions are the same as those in the HLM–LC–MS assay, except the rhCYP cocktail was used instead of the HLM.

2.7. HLM stability assay

Microsomal stability of each test compound was determined using the method reported previously (Di et al., 2003, 2004, 2006) and the same HLM batch as in the inhibition studies. An incubation time of 20 min was used for the microsomal stability assay to be consistent with the incubation time used in the CYP450 inhibition assay. The final microsomal protein concentration was 0.5 mg/mL. The purpose was to determine the % remaining of test compounds to indicate the actual concentrations of test compounds during the assay.

3. Results and discussions

A disparity in results from the rhCYP-fluorescent and HLM–LC–MS assays has been noted, but the causes of the difference would benefit from further evidence. This study examined these differences and investigated the experimental differences that could be responsible for the disparity. In addition, results from the double cocktail assay, which has the advantages of cocktail drug-probe substrates and cocktail rhCYP isozymes, were determined for the same test compounds.

3.1. Comparison of data for drug discovery compounds with the rhCYP-fluorescent and HLM–LC–MS assays

Over 60 structurally diverse Wyeth Research drug discovery compounds were assayed using the rhCYP-fluorescent and HLM–LC–MS assays. The data are shown Figs. 1–3. For the rhCYP-fluorescent assay, the percent inhibition at 3 μM was determined, which has been shown to have good correlation with IC_{50} (Gao et al., 2002). The IC_{50} was then determined for the same compounds using the HLM–LC–MS assay. The results show there is a general trend of correlation between the two assays. However, the data are quite scattered. The correlation coefficients (R^2) were: 0.15 for 3A4, 0.34 for 2D6 and 0.24 for 2C9. For CYP3A4 and CYP2C9, there was a large group of compounds that showed high inhibition in the rhCYP-fluorescent assay (high % inhibition) but low inhibition in the HLM–LC–MS assay (high IC_{50}), which is the "HL" region in the figures. However, there were no compounds that showed high inhibition in the HLM–LC–MS assay, and low inhibition in the rhCYP-fluorescent assay ("LH" region in the figures). This suggests that the HLM–LC–MS assay tends to give higher IC_{50} than the rhCYP-fluorescent assay. CYP2D6 has the strongest correlation between the two assays. There are fewer compounds in the "HL" region for 2D6 than for 3A4 and 2C9. However, the data are still quite scattered.

3.2. Comparison of data for commercial drug compounds with the three assays

IC_{50}s of commercial drugs using all three assays are tabulated in Tables 4–6. Similar trends to the Wyeth Research discovery compounds for data between the rhCYP-fluorescent and HLM–LC–MS methods were observed for the commercial drugs. IC_{50}s from the HLM–LC–MS assay were higher than those from the rhCYP-fluorescent assay. These results are in agreement with those reported by other investigators, with CYP3A4 having the weakest correlation, 2D6 having the best correlation and the overall correlation between the two assays being weak (Favreau et al., 1999; Nomeir et al., 2001; Cohen et al., 2003). Data from the double cocktail assay agreed most closely with data from the rhCYP-fluorescent assay.

3.3. Causes of differences between the assays

A study was undertaken of the potential methodological causes of disparity in data between the assays. There are three major differences between the rhCYP-fluorescent and HLM–LC–MS assays (Table 3, Fig. 4): (1) detection techniques

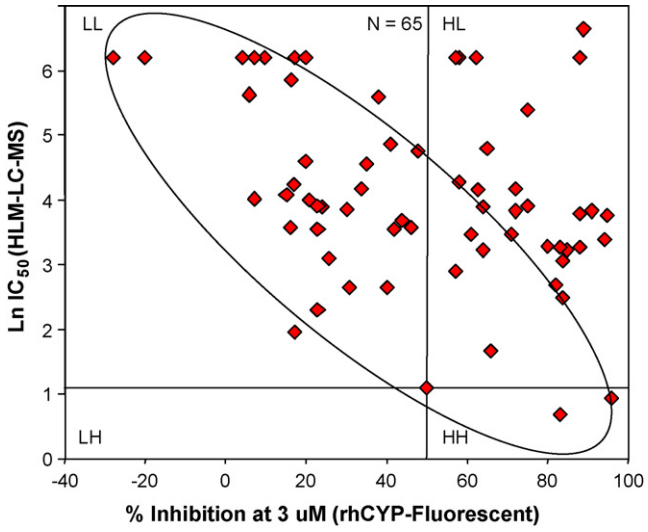

Fig. 1. Comparison of CYP3A4 inhibition between rhCYP-fluorescent and HLM–LC–MS assays for 65 Wyeth research discovery compounds.

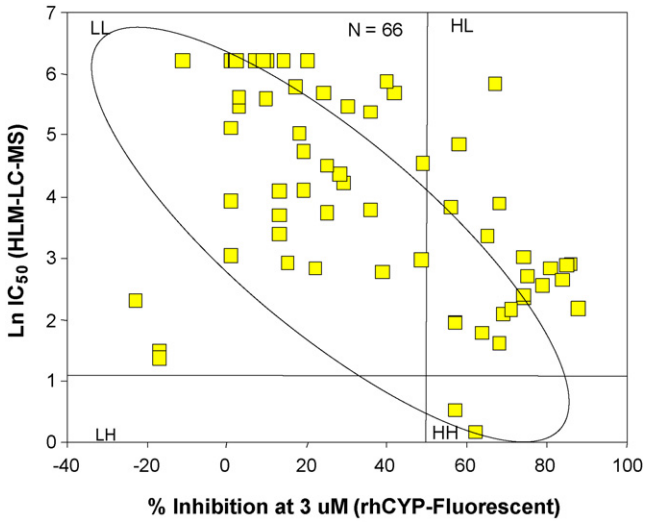

Fig. 3. Comparison of CYP2C9 inhibition between rhCYP-fluorescent and HLM–LC–MS assays for 66 Wyeth research discovery compounds.

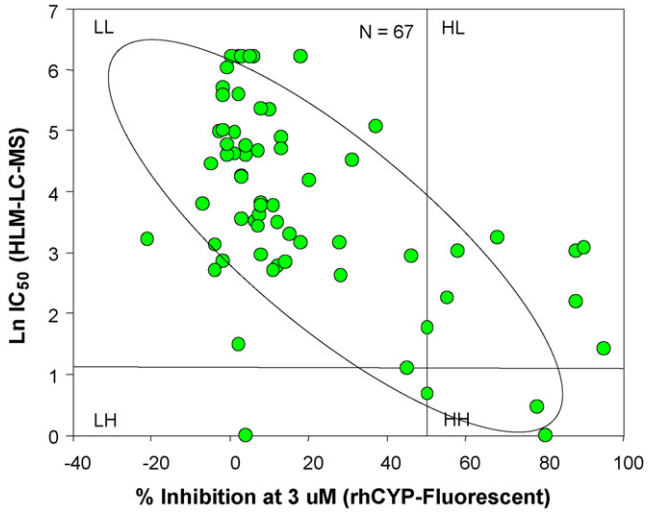

Fig. 2. Comparison of CYP2D6 inhibition between rhCYP-fluorescent and HLM–LC–MS assays for 67 Wyeth research discovery compounds.

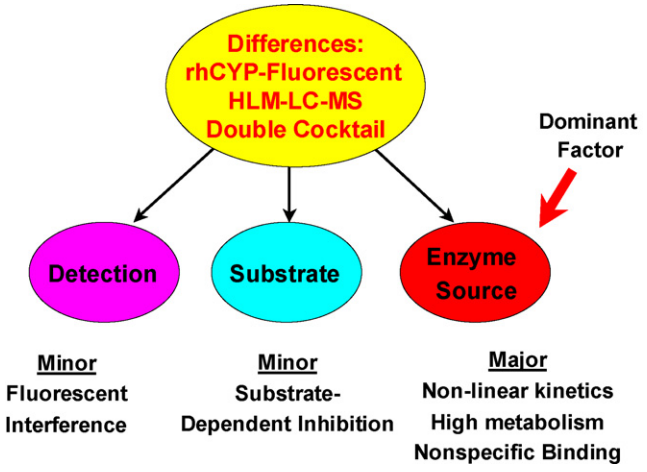

Fig. 4. Differences between rhCYP-fluorescent, HLM–LC–MS and double cocktail assays.

(fluorescent versus LC–MS), (2) substrates (coumarin derivative probe substrates versus drug-probe substrates), and (3) enzyme source (rhCYP450 isozymes versus human liver microsomes) and composition/concentration. These were investigated for their effects on IC_{50}.

3.3.1. *Effects of detection techniques and substrates*

Fluorescent detectors have the advantages of high throughput, high capacity, high speed and high sensitivity. They are fully amenable to integration with robotic systems in high throughput settings. Each 96-well plate takes about 1 min to read (~0.6 s/well), which makes the fluorescent plate reader one of the fastest detectors. Fluorescent detectors also require minimal maintenance and less experienced operators. Most discovery compounds are suitable to be used with fluorescent detection assays, unless the compound has fluorescent interference, due to auto-fluorescence, color quenching or light scattering after precipitation (Zou et al., 2002). In these cases, both false positive and false negative results can be generated. If the metabolites

Table 3
Differences between the CYP450 inhibition assays

Assay	rhCYP-fluorescent	HLM–LC–MS	Double cocktail
Detection	Fluorescent	LC–MS–MS	LC–MS–MS
Substrate	Coumarin derivative probe individually	Drug-probe cocktail	Drug-probe cocktail
Enzyme source	rhCYP individually	Human liver microsomes	rhCYP cocktail

generated from the test compounds are fluorescent, they can interfere with the assay and lead to false negative results. Certain classes of compounds are not suitable for the fluorescent assay due to interference (Vaz, 2005). Because of the potential false negative results, FDA does not recommend using the fluorescent assay for regulatory submission. Nevertheless, the fluorescent assay is still a useful tool in early drug discovery, due to its throughput and low maintenance, as long as the users are fully aware of the potential limitations.

LC–MS has the advantages of high specificity and it is unlikely to have interference from test compounds or metabolites. The specific metabolite generated from each probe substrate is selectively detected. Therefore, a cocktail method with multiple probe substrates can be used with LC–MS. Several metabolites from specific probe substrates can be monitored simultaneously to determine the inhibition of several CYP450 isozymes in one LC–MS analysis (Dierks et al., 2001; Bu et al., 2000; Zhang et al., 2002; Testino et al., 2003). The major limitation of LC–MS is throughput. It takes about 3 min/well for analysis, which is about 300-fold slower than using the fluorescent detector. LC–MS also requires high maintenance, a more experienced operator, and consumes more solvent.

If a compound has fluorescence interference (Zou et al., 2002), the fluorescent and LC–MS assays will give different results. However, for most of the compounds that do not have fluorescent interference, the two assays should give similar assay results regardless of which detection technique is used, whether fluorescent or LC–MS.

The substrates used in the two assays are shown in Table 1. They are very different. One assay uses coumarin-derivative substrates and the other uses drug-probe substrates. Substrate-dependent inhibition has been reported in several studies (Stresser et al., 2000; Wang et al., 2000; Kenworthy et al., 1999; Galetin et al., 2005). It has been shown that the CYP3A4 IC_{50} can vary from 2.1- to 195-fold, with an average of 29-fold, when using four different fluorescent substrates for 21 inhibitors (Stresser et al., 2000). Because of the large binding pocket of CYP3A4, different substrates can have different binding sites and lead to different inhibition results. Another study showed that Vivid Red does not reflect the interaction of compounds with the CYP3A4 active site that is observed for testosterone (Cohen et al., 2003). PhRMA recommends using multiple substrates for CYP3A4 inhibition studies, due to the large binding pocket of this enzyme (Bjornsson et al., 2003; Tucker et al., 2001).

If the different detection techniques or substrates are responsible for the data difference, this should be indicated by comparison of data from the rhCYP-fluorescent and double cocktail methods. The same commercial drugs were assayed using the two methods. Differences in the assays were: the probe substrates (coumarin derivatives versus drugs) and detection (fluorescence versus LC–MS). The same enzyme source (rhCYP isozymes) were used in both methods. Fig. 5 shows a comparison between the two data sets. The data have excellent agreement, with $R^2 > 0.94$. Therefore, the differences in detection techniques and substrates do not appear to be the major causes of disparity in assay results.

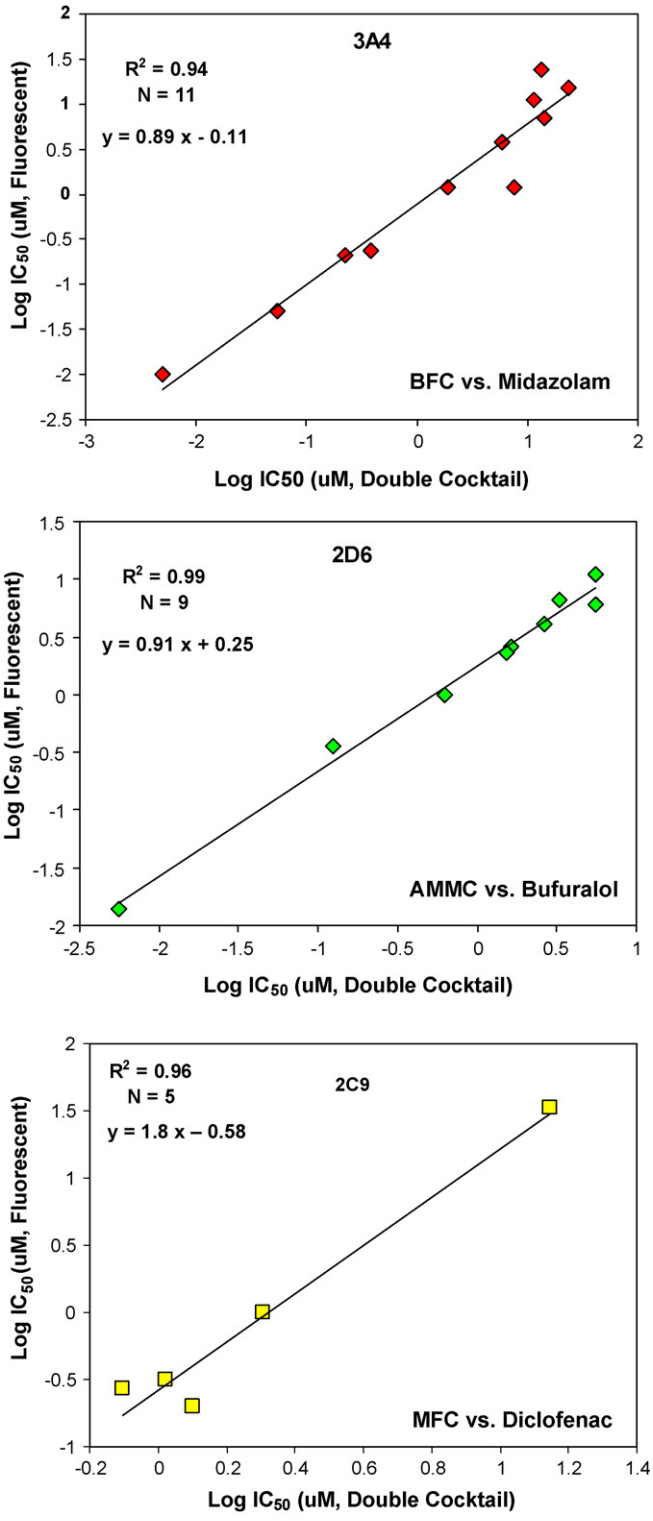

Fig. 5. Comparison of IC_{50}s for test compounds in Tables 4–6 using rhCYP-fluorescent assay vs. double cocktail assay.

3.3.2. *Effects of enzyme source and composition*

Enzyme source and composition is another major difference between the assays. In the rhCYP-fluorescent and double cocktail assays, purified cDNA-expressed rhCYP450 isozymes are used. The concentrations of each isozyme in the assay are shown

in Table 1 and can be independently changed to optimize the enzymatic conditions of the assay.

In the HLM–LC–MS assay, human liver microsomes are used. The concentrations of individual CYP450 isozymes in the HLM vary greatly, as shown in Table 1. These isozyme concentrations are fixed by their natural abundance in liver tissue. At a protein concentration of 0.5 mg/mL, the CYP450 isozyme concentrations from HLM in the HLM–LC–MS assay are much higher than in the rhCYP-fluorescent assay. The 3A4 isozyme concentrations are 42 pmol/mL in HLM–LC–MS assay and 5 pmol/mL rhCYP-fluorescent assay, respectively. 2D6 concentrations are comparable. 2C9 concentrations are also higher in the HLM–LC–MS assay (37 pmol/mL in the HLM–LC–MS assay and 20 pmol/mL rhCYP-fluorescent assay, respectively).

Table 4 shows the IC_{50} values of a set of commercial drugs for CYP3A4. The IC_{50}s in the HLM–LC–MS assay were at least 10- to 100-fold higher than those in the rhCYP-fluorescent assay, when the protein concentration was 0.5 mg/mL. When protein concentration was reduced to 0.1 mg/mL, most of the IC_{50}s dropped and were closer to those in the rhCYP-fluorescent assay, but they are still higher. This is consistent with the results obtained from the 65 Wyeth Research discovery compounds shown in Fig. 1, with the rhCYP-fluorescent assay showing higher inhibition than the HLM–LC–MS assay.

The data for % conversion of probe substrates (last row in each table) and % remaining of the test compounds (last column in each table) in Tables 4–6 suggest three reasons for the high IC_{50}s from the HLM–LC–MS assay, as compared to those from the rhCYP-fluorescent assay. Firstly, there is high substrate conversion for the CYP3A4 drug-probe substrates by the HLM. For CYP3A4, the substrate conversion of midazolam was 35% at 0.5 mg/mL HLM protein concentration and 16% at 0.1 mg/mL HLM protein concentration. This high substrate conversion will lead to non-linear enzyme kinetics and unreliable assay results, because the kinetic equation based on initial rate does not hold under these conditions. It is recommended that incubation conditions produce a minimum of substrate conversion (<10%) to assess the initial linear kinetics and minimize potential metabolic product inhibition (Bjornsson et al., 2003; Cohen et al., 2003; Tucker et al., 2001; Rodrigues, 1999). Secondly, high metabolism of many test compounds occurred because of the high enzyme concentrations in the HLM–LC–MS assay. The percent remaining (unmetabolized) of each test compound under the incubation conditions is shown in Table 4. Many test compounds were highly metabolized. This will cause inhibitor depletion and the actual concentration in the assay will be lower than the target (putative) concentration. High metabolism of test compound will lead to inhibitor depletion and result in an artificially high IC_{50} (Tran et al., 2002; Gibbs et al., 1999). Thirdly, high non-specific binding of test compounds to phospholipids and proteins in HLM have been shown to reduce the effective concentration of test compound in the assay and generate high IC_{50}s (Walsky and Obach, 2004; Margolis and Obach, 2003).

Table 5 shows a comparison of the two assays for CYP2D6. Because of the naturally low abundance of this enzyme in HLM (∼2%), the conversion rate of substrate was 4% for bufuralol, which was much lower than the 35% conversion rate of midazolam substrate in the CYP3A4 HLM–LC–MS assay. Most test compounds (inhibitors) also had low metabolism during incubation. The IC_{50}s in the HLM–LC–MS assay were similar to those in the fluorescent assay when protein concentration was 0.1 mg/mL. These results for 2D6 are consistent with the results obtained for 2D6 from 67 Wyeth compounds (Fig. 2). CYP2D6 showed the strongest correlation between the two assays, due to the low amount of this isozyme in the assay.

Table 6 shows a comparison of the two assays for CYP2C9. Similar to CYP3A4, CYP2C9 had high substrate conversion (40% at 0.5 mg/mL protein concentration and 23% at 0.1 mg/mL) and high metabolism of test compounds (inhibitors). The IC_{50}s from the HLM–LC–MS assay were 2- to 50-fold

Table 4
CYP3A4 inhibition IC_{50} and microsomal stability of test compounds and % conversion of probe compound with different assays

Assay	HLM–LC–MS	HLM–LC–MS	rhCYP-fluorescent	Double cocktail	Human liver microsomal stability
Total protein concentration	0.5 mg/mL	0.1 mg/mL	NA	NA	0.5 mg/mL
3A4 concentration	42 pmol/mL	8.4 pmol/mL	5.0 pmol/mL	0.78 pmol/mL	42 pmol/mL
Probe substrate	Midazolam	Midazolam	BFC	Midazolam	NA
Test compound	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	% remaining of test compound at 20 min
Clotrimazole	0.46	0.041	<0.01	0.005	25
Ethynylestradiol	>90	>100	1.2	7.6	0
Miconazole	5.4	0.49	0.21	0.23	43
Nicardipine	>20	2.1	0.24	0.39	3
Fluconazole	>100	19	24	13	97
Terfenadine	>20	18	1.2	1.9	58
Verapamil	>40	26	3.9	5.9	21
Erythromycin	>50	28	15	23	65
Nifedipine	>100	24	11	12	10
Clomipramine	>100	28	6.9	14	87
Ketoconazole	0.60	0.14	0.05	0.05	89
% conversion of probe substrate	35%	16%	NA	1%	NA

NA: not applicable.

Table 5
CYP2D6 inhibition IC_{50} and microsomal stability of test compounds and % conversion of probe compound with different assays

Assay	HLM–LC–MS	HLM–LC–MS	rhCYP-fluorescent	Double cocktail	Human liver microsomal stability
Total protein concentration	0.5 mg/mL	0.1 mg/mL	NA	NA	0.5 mg/mL
2D6 concentration	4.2 pmol/mL	0.84 pmol/mL	7.5 pmol/mL	0.28 pmol/mL	4.2 pmol/mL
Probe substrate	Bufuralol	Bufuralol	AMMC	Bufuralol	NA
Test compound	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	% remaining of test compound at 20 min
Miconazole	7.0	3.0	2.6	1.6	43
Nicardipine	15	5.9	6.1	5.6	3
Fluvoxamine	18	5.4	4.1	2.6	94
Imipramine	18	3.5	6.6	3.3	90
Clomipramine	12	0.33	2.3	1.5	87
Fluoxetine	0.76	0.15	1.0	0.62	104
Timolol	35	3.4	11	5.6	92
Yohimbine	0.06	0.05	0.36	0.13	80
Quinidine	0.03	0.05	0.01	0.01	94
% conversion of probe substrate	4%	1%	NA	2%	NA

NA: not applicable.

higher than those from the rhCYP-fluorescent assay. This is consistent with the results obtained from 66 Wyeth compounds (Fig. 3).

The data are in agreement with the observation that the HLM–LC–MS assay has higher IC_{50}s than the rhCYP-fluorescent assay, especially for CYP3A4 and 2C9. The study indicates that the major limitations of using a high amount of HLM in CYP450 inhibition studies are high substrate conversion and high metabolism of test compounds (high non-specific binding is likely also a factor, but was not specifically tested in this study). This is even more challenging for cocktail approaches with HLM, because the relative concentration of isozymes cannot be adjusted in the assay. The concentrations of the individual enzymes can only be increased or decreased at the same time, but the ratio among the different isozymes stays the same. If protein concentration is high, it can be too high for linear enzyme kinetics for the most abundant isozymes (e.g., CYP3A4, CYP2C9). However, if the protein concentration is low, it can be too low for detection of substrate metabolites produced by the low abundance isozymes (e.g., CYP2D6, CYP1A2).

This analysis indicates that differences in enzyme source and composition/concentration are the dominant factors that cause the differences in inhibition data between rhCYP-fluorescent and HLM–LC–MS assays (Fig. 4).

3.4. Double cocktail assay

Studies using the double cocktail assay in parallel with the above experiments indicate that it can overcome the limitations of the current assays. Weaver et al. (2003), reported using a mixture of rhCYP450 isozymes with a mixture of drug-probe substrates. This allows selection of the most desirable isozyme concentrations, in order to achieve optimal conditions for enzyme kinetics. We have modified and adapted the double cocktail assay (1st cocktail: mixture of rhCYP450 isozymes, second cocktail: mixture of drug-probe substrates) using concentrations of each isozyme and substrate as shown in Tables 1 and 2. The assay uses a very small amount of each CYP450 isozyme. This achieves the low drug-probe substrate conversion rate of 1–2%, as shown in Tables 4–6. The low probe conver-

Table 6
CYP2C9 inhibition IC_{50} and microsomal stability of test compounds and % conversion of probe compound with different assays

Assay	HLM–LC–MS	HLM–LC–MS	rhCYP-fluorescent	Double cocktail	Human liver microsomal stability
Total protein concentration	0.5 mg/mL	0.1 mg/mL	NA	NA	0.5 mg/mL
2C9 concentration	37 pmol/mL	7.4 pmol/mL	20 pmol/mL	0.55 pmol/mL	37 pmol/mL
Probe substrate	Diclofenac	Diclofenac	MFC	Diclofenac	NA
Test compound	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	% remaining of test compound at 20 min
Clotrimazole	6.8	2.1	0.2	1.3	25
Miconazole	8.0	2.4	1.0	2.0	43
Nicardipine	15	4.9	0.32	1.1	3
Nifedipine	28	10	33	14	10
Sulfaphenazole	0.75	0.41	0.27	0.79	90
% conversion of probe substrate	40%	23%	NA	1%	NA

NA: not applicable.

sion rate accesses the initial rate of enzyme inhibition kinetics. Metabolism of test compounds (inhibitors) will also be minimized at this low isozyme concentration. LC–MS detection provides high selectivity for the drug-probe substrate metabolites (Table 2) and eliminates fluorescent interference issues of the fluorescence detection method.

The results obtained from the double cocktail assay for the commercial drugs are shown in Tables 4–6. Strong correlation of IC_{50}s from the rhCYP-fluorescent and double cocktail assay are shown in Fig. 5. The two assays have excellent correlation.

In cocktail analysis methods, it is always important to check for interference among the cocktail components. This was studied by incubating each commercial drug in Tables 4–6 with the corresponding individual drug-probe substrate and its corresponding individual CYP isozymes. Data for this study are shown in Fig. 6 and indicate a high correlation between the double cocktail assay results and the individual (discrete) assays. This suggests that the double cocktail assay increases throughput without sacrificing data quality.

The advantages of the double cocktail assay are: (1) high assay sensitivity (low IC_{50}) compared to assays using HLM, and (2) no interference compared to fluorescent assays, due to auto-fluorescence or fluorescent quenchers. The assay is high throughput by monitoring inhibition of several isozymes at the same time (in one well, with one LC–MS analysis). The double cocktail assay has lower costs than the current assays, because it uses a very small amount of enzyme.

3.5. CYP450 inhibition assay selection in drug discovery

In drug discovery, CYP450 inhibition data are used to provide early alerts to potential issues, prioritize chemical series, and guide SAR to overcome CYP inhibition. For compounds that are known to not have fluorescent interference, the fluorescent assay can rapidly provide CYP inhibition data for discovery teams, due to the high assay throughput. Compounds for which fluorescent interference is encountered, or if only one assay can be run and fluorescent interference is unknown, the double cocktail assay can reliably provide CYP inhibition data. The double cocktail assay throughput is lower than the rhCYP-fluorescent assay, but it is reasonable (comparable to many other in vitro ADMET assays).

HLM is a complex system for a CYP inhibition assay. In addition to inhibition, HLM assays also have non-CYP inhibition activities that contribute to the final assay data. These activities include: metabolism (substrate and inhibitor, as shown in Tables 4–6), protein binding, and non-specific lipid binding. These activities affect the results and produce an apparent inhibition result, rather than an intrinsic inhibition result. Assays using rhCYP450s with very low protein concentration minimize the side (non-CYP inhibition) activities of metabolism and binding to assay matrix materials (protein and lipid), which affect and confuse CYP inhibition study results. When low protein concentration is used, the data are more reliable for the actual (intrinsic) IC_{50}. A cocktail approach with HLM is difficult, because the low abundance CYP isozymes require a higher protein concentration, in order to detect the substrate metabolite produced by

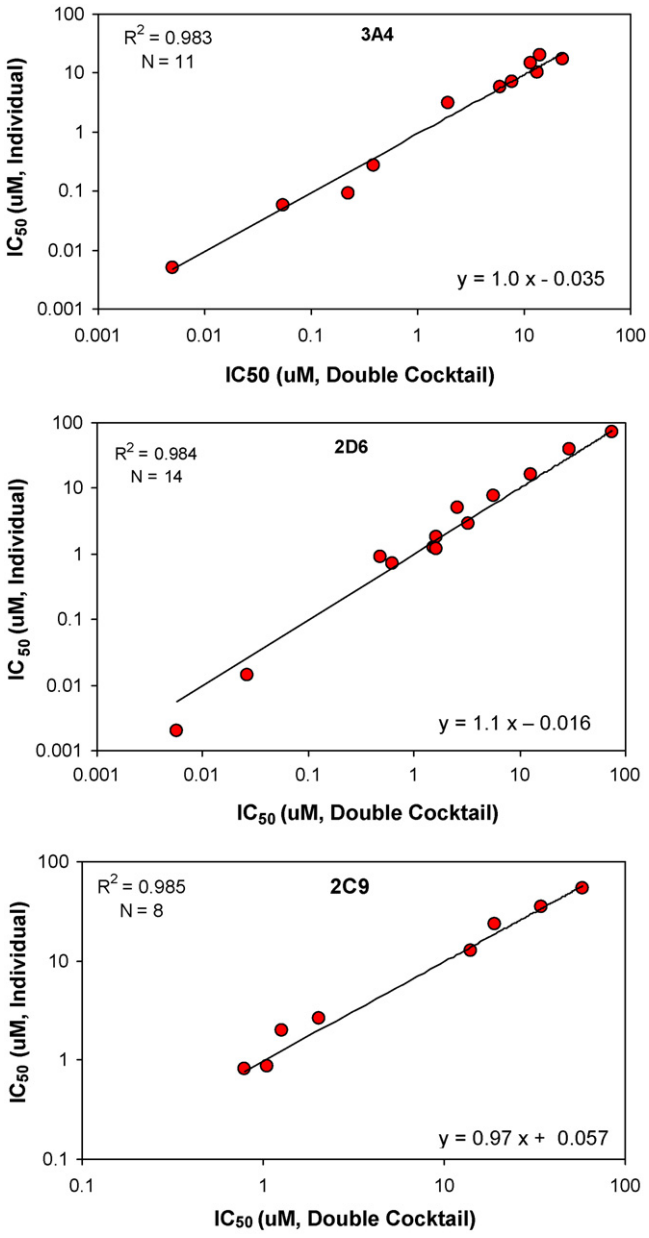

Fig. 6. Correlation of IC_{50}s between double cocktail assay and assay of individual drug-probe substrates with their respective individual isozyme.

the reaction. However, these higher protein concentrations also cause high concentrations of the high abundance CYP isozymes, which results in non-linear kinetics for their substrates and the test compounds. It has been reported that, for development candidates, using HLM at low concentrations in a non-cocktail assay that is optimized for each substrate-isozyme pair, can provide reliable data (Walsky and Obach, 2004). This approach is too low throughput for drug discovery.

It has been argued that HLM is more physiologically relevant than rhCYP isozymes, because of the side activities of metabolism and binding. However, if the assay design is not optimal for enzyme kinetics, the high substrate conversion, high metabolism of inhibitors and high non-specific binding will yield unreliable and high apparent IC_{50}s. CYP inhibition is one

of many selectivity issues that drug discovery scientists must deal with and selectivity assays are typically performed in an assay that is not complicated by compound metabolism and non-specific binding, in order to measure intrinsic IC_{50}.

4. Conclusions

The rhCYP-fluorescent CYP450 inhibition assay has the advantages of high assay sensitivity, high throughput, and low maintenance. However, the assay is not suitable for compounds that have fluorescent interference. The HLM–LC–MS assay tends to have high IC_{50}s when microsomal protein concentration is too high. Systems using HLM have complex mechanisms and it is difficult to use them to guide medicinal chemistry SAR in drug discovery. The resulting IC_{50} is affected by the high substrate conversion, inhibitor depletion, and non-specific binding, while medicinal chemists need intrinsic IC_{50}s to guide lead selection and structure modification. rhCYP-fluorescent and HLM–LC–MS CYP450 inhibition assays have weak correlations when there are major differences in enzyme source and enzyme concentration. The double cocktail assay has advantages of both assays and does not have the limitations of either assay. It has high assay sensitivity and no fluorescent interference. The assay can be used in drug discovery and development to address drug–drug interaction issues and develop SAR to circumvent CYP450 inhibition.

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