Evaluation of High Throughput Screening Methods for Time-Dependent Inhibition of CYP3A4 Utilizing RapidFire LC/MS/MS Technology

Vaughn P. Miller[1], Can C. Ozbal[1], Elke S. Perloff[2], Shangara S. Dehal[2], Andrew K. Mason[2], Andrew P. Blanchard[2], Charles L. Crespi[2], David M. Stresser[2], and William A. LaMarr[1]

[1] **BioTrove, Inc.**, 10P Gill St. Woburn, MA 01801, USA; [2] **BD Biosciences**, 6 Henshaw Street, Woburn, MA 01801, USA

Introduction

Time-dependent inhibition (TDI) of cytochrome P450 can cause clinically relevant drug-drug interactions (DDI) and lead to termination of development, withdrawal from the market or severe restriction on the use of new therapies. Recently, there has been a greater recognition of the importance of screening for TDI of human cytochrome P450s earlier in drug discovery. The increased interest in TDI screening has been facilitated by recent advances in understanding how *in vitro* TDI data can be used to predict clinical DDI. Several reviews of TDI and *in vitro* methodology have been published.[1-4]

The increased interest in TDI has lead to the demand for higher throughput analysis options for this assay. Today in drug discovery, these assays are most commonly done using human liver microsomes (HLMs) and LC/MS analysis of drug probe substrates in assays that examine a shift in IC_{50} or the change in the amount of inhibition at 1-2 inhibitor concentrations.[1,4] In discovery, the TDI of CYP3A4 is usually evaluated due to it's importance in drug clearance.[1]

The objective of this study was to compare the results from RapidFire® LC/MS/MS technology to traditional LC/MS/MS analysis for *in vitro* CYP3A4 TDI assays to assess the potential for the high throughput RapidFire technology to be utilized for these important drug discovery assays. We also reviewed the data for a comparison of single versus multi-concentration methods for *in vitro* CYP3A4 TDI screening.

Methods

7-point IC_{50} values for a range of inhibitors were determined using the standard dilution approach in duplicate individual incubations in HLMs using FDA preferred CYP3A4 drug probe substrates (midazolam and testosterone) with previously validated assay methods,[2,5] which are summarized in **Table 1**. Stable-labeled isotope internal standards (BD Biosciences, Woburn, MA) were used for both probe substrate metabolites.

Samples were analyzed individually by RapidFire technology as well as by traditional LC/MS/MS methods. Traditional LC/MS/MS methods were developed and validated at BD Biosciences using an Applied Biosystems API4000 instrument and a 2-4 min solvent gradient on a Waters® Symmetry® C18 column.[2] RapidFire ultra high throughput LC/MS/MS methods were developed and run on a BioTrove RapidFire microscale solid-phase extraction preparation system interfaced to an ABI-4000 triple quadrapole mass spectrometer with an average sample cycle time of 7 seconds. RapidFire MS methods are shown below in **Table 2**. Both assays were analyzed in positive ion mode using a mobile phase of 0.09% formic acid and 0.01% TFA in acetonitrile and RapidFire column A.

Percent remaining activity was calculated for each inhibitor concentration and IC_{50} values were determined by linear interpolation.

T1 Incubation Conditions

Enzyme	Substrate	Time Dependent Inhibition HLM concentration (mg/mL)*	Incubation time	Substrate concentration used [μM]
CYP3A4	Midazolam	0.2 → 0.02	5 min	3.0
CYP3A4	Testosterone	0.5 → 0.05	10 min	50

* HLM concentrations used in the pre-incubations (to the left of the arrow) and after dilution into secondary incubations (to the right of the arrow). For direct inhibition protocols, protein concentrations were equivalent to diluted concentrations (right).

T2 RapidFire MS Methods

	Internal Standard			Product				
CYP Assay		Q1	Q3		Q1	Q3	CE (eV)	DP (eV)
CYP3A4	1´-Hydroxymidazolam-[$^{13}C_3$]	345.0	206.1	1´-Hydroxymidazolam	342.0	203.1	35	80
CYP3A4	6 -Hydroxytestosterone-[D_7]	312.2	275.8	6 -Hydroxytestosterone	305.3	269.5	22	85

Results

IC_{50} values obtained using RapidFire ultra high throughput LC/MS/MS analysis were consistent with the data obtained using traditional LC/MS/MS methods validated at BD Biosciences (R^2 = 0.999, **Figure 2**). 90% of corresponding IC_{50} values were within 1.2 fold of each other and 100% were within 1.9 fold of each other (**Table 2**).

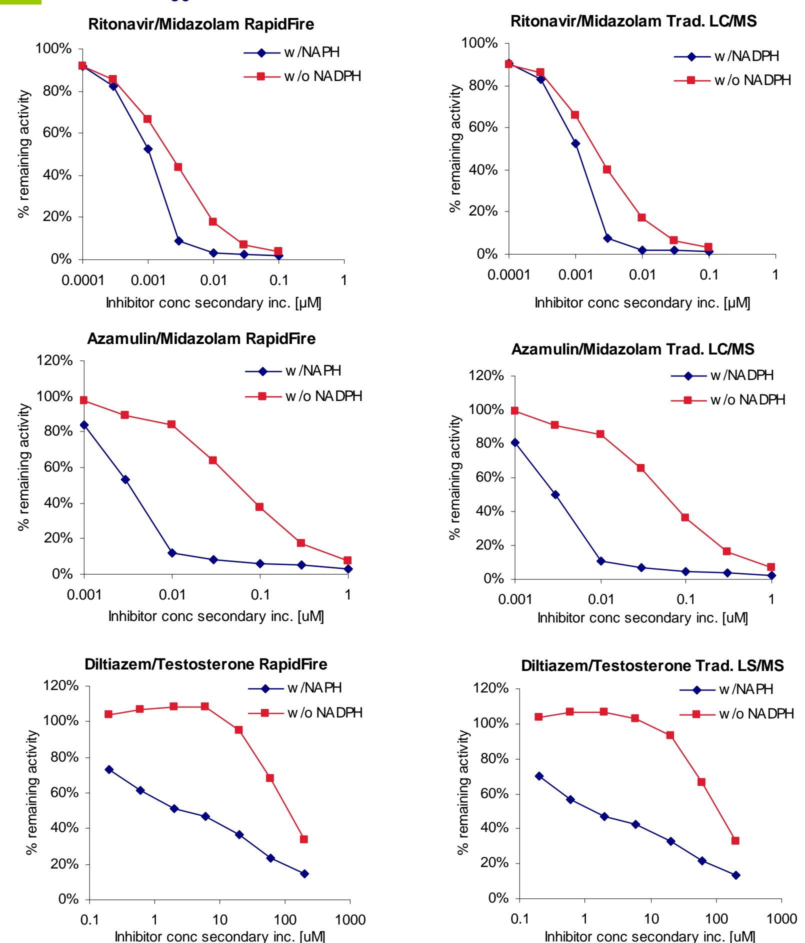
A run time of approximately 7 seconds per cycle with RapidFire technology compared to 2 to 4 minutes for traditional LC/MS/MS methods provided a significant (> 20-fold) decrease in analysis time. A full IC_{50} curve (7 pts) can be analyzed in less time than a single data point using traditional LC/MS/MS methods.

Reviewing the data from these known CYP3A4 TDI inhibitors, it can be seen that an experiment that looks at only the change in the amount of inhibition at 1-2 inhibitor concentrations (10-50 μM) would show significant inhibition for all of these compounds and could be useful for initial screening. However, for compounds with IC_{50} values substantially different than the single concentration used (i.e. strong inhibitors like ritonavir and azamulin; or very weak inhibitors (IC_{50} > 100 μM)), it may be difficult to distinguish between direct inhibition and TDI under these conditions because the IC_{50} curves will have either converged or be very close together at these single concentrations. The IC_{50} shift methodology, while more resource intensive, provides a data rich assay for decision making.

References

1. Grimm, S.W., et al., *Drug Metab. Disp.* Fast Forward DOI:10.1124/dmd.109.026716 (2009).
2. Perloff, E.S., et al., *Xenobiotica* **39**:99 (2009).
3. Obach, R.S., et al., *Drug Metab. Disp.* **35**:246 (2007).
4. Riley, R.J., et al., *Expert Opin Drug Metab. Toxicol.* **3**:51 (2007).
5. Draft Guidance for Industry: Drug Interaction Studies — Study Design, Data Analysis, and Implications for Dosing and Labeling (www.fda.gov/cder/guidance/6695dft.pdf)

F1 Select IC_{50} Curves



T3 IC_{50} Values

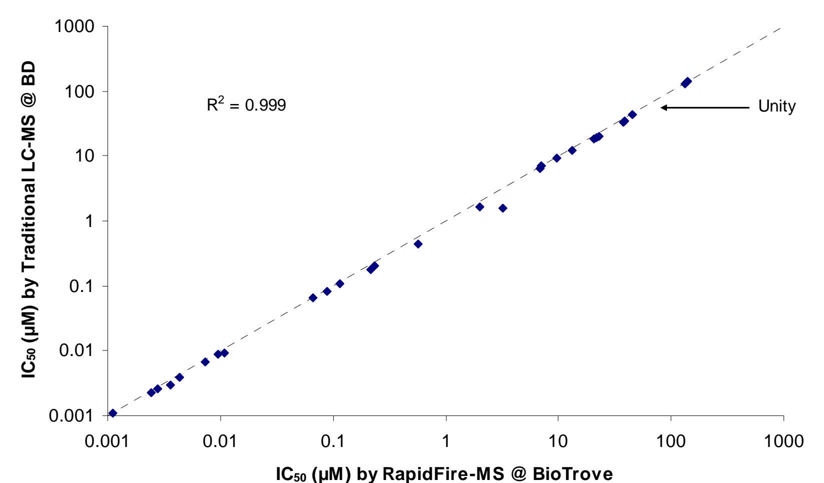
Midazolam

	RapidFire-MS			Trad. LC-MS BD			Ratio Trad./RapidFire		
Compound	w/ NADPH	w/o NADPH	Shift	w/ NADPH	w/o NADPH	Shift	w/ NADPH	w/o NADPH	Shift
Tamoxifen	7.106	6.790	0.96	6.955	6.55	0.94	0.98	0.96	0.99
Fluoxetine	37.428	38.52	1.03	32.938	33.90	1.03	0.88	0.88	1.00
Ritonavir	0.001	0.002	2.17	0.001	0.002	2.03	0.98	0.92	0.93
Diltiazem	13.295	141.26	10.63	11.951	139.21	11.65	0.90	0.99	1.10
Azamulin	0.004	0.066	18.41	0.003	0.066	22.23	0.83	1.01	1.21
Erythromycin	2.002	45.72	22.83	1.629	44.38	27.24	0.81	0.97	1.19
Mibefradil	0.003	0.089	31.58	0.003	0.083	31.32	0.94	0.94	0.99
Verapamil	0.231	20.65	89.28	0.205	18.44	89.77	0.89	0.89	1.01

Testosterone

	RapidFire-MS			Trad. LC-MS BD			Ratio Trad./RapidFire		
Compound	w/ NADPH	w/o NADPH	Shift	w/ NADPH	w/o NADPH	Shift	w/ NADPH	w/o NADPH	Shift
Fluoxetine	9.686	22.91	2.37	9.242	20.09	2.17	0.95	0.88	0.92
Ritonavir	0.004	0.011	2.47	0.004	0.009	2.40	0.89	0.86	0.97
Azamulin	0.010	0.116	12.05	0.009	0.107	12.06	0.93	0.93	1.00
Diltiazem	3.250	133.36	41.04	1.601	126.92	79.26	0.49	0.95	1.93
Mibefradil	0.007	0.57	78.98	0.007	0.441	65.37	0.93	0.77	0.83
Verapamil	0.216	21.55	99.77	0.181	19.62	108.37	0.84	0.91	1.09
Tamoxifen	> 100	> 100	N/A	> 100	> 100	N/A	N/A	N/A	N/A
Erythromycin	1.582	> 100	>60	1.12	> 100	>90	0.71	N/A	N/A

F2 Correlation between RapidFire-MS and Traditional LC-MS



Conclusions

- Results from the RapidFire LC/MS/MS technology for the analysis of *in vitro* CYP3A4 TDI screening were comparable to traditional LC/MS/MS methods (90% of values were within 1.2 fold of each other, 100% within 1.9 fold, R^2 = 0.999).
- The data in this study suggests that a single inhibitor concentration provides relevant outcomes for initial screening. However, it would be difficult to distinguish between TDI and direct inhibition for some compounds (those whose IC_{50} values are much larger or smaller than the concentration tested) using this method alone.
- As expected, full IC_{50} shift determinations improve robustness of TDI determination, but increase sample load. However, the > 20-fold decrease in assay analysis times using the RapidFire system allow for the analysis of a full IC_{50} curve in the time traditionally spent analyzing a single data point. This great increase in speed should offset concerns with the turnaround time required for full inhibition curves for this important ADMET screen.

For Research Use Only. Not intended for use in diagnostic or therapeutic procedures.
RapidFire is a registered trademark of BioTrove, Inc.
Symmetry and Waters are registered trademarks of Waters Corp.
BD, BD Logo, and all other trademarks are the property of Becton, Dickinson and Company. ©2009 BD