



Development of a fluorimetric assay to identify inhibitors of recombinant CYP2B6

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Introduction

Here we describe the validation and testing of our latest assay to investigate the inhibitory potential of 27 compounds on the catalytic activity of recombinant CYP2B6 coexpressed with NADPH-CYP reductase and supplemented with cytochrome b₅ (product code CYP/EZ041). This is useful information to have fairly early in the drug discovery/lead optimisation process as it can differentiate between compounds which are likely to have significant effect on drug-drug interactions before a lead compound reaches the stages of conducting clinical trials. Our method describes a fluorimetric assay which is relatively quick, easy and cost-effective to perform whilst also being predictive of the end result *in vivo*.

Method

The substrate 7-EFC (7-ethoxy-4-trifluoromethylcoumarin) is metabolised to a fluorescent metabolite 7-HFC (7-hydroxy-4-trifluoromethylcoumarin) by CYP2B6 with an apparent K_m for the reaction of 1.7-1.9 µM and V_{max} of 3100-3400 RFU/min⁻¹ between 2 batches of enzyme irrespective of whether DMSO or methanol was used as solvent at a final concentration of 2% (v/v). The 7-HFC production was monitored at excitation and emission wavelengths of 431 and 535 nm, respectively using a Tecan Infinite M200 instrument running Magellan software (v6.2, Reading, Berkshire, UK), formation of metabolite was linear for approximately 20 mins but slowed down at later time-points during a 30 minutes incubation period at 37°C.

27 compounds were investigated to calculate IC₅₀ values against this enzymatic reaction using the above detection methods; potential inhibitors were serially diluted and assayed at final incubation concentrations over the 100-0.14 µM range or lower depending where the LLQ for the assay lay. Both DMSO and methanol were present as solvents accounting for either 1.5% or 2.5% (v/v) of the final incubation volume of 250 µL (solvents were matched between substrate and potential inhibitors).

5 µL of each potential inhibitor was applied to a black polypropylene 96-well plate (Greiner 655209) before 220 µL premix (comprising of KPO₄ [50 mM, pH 7.4 containing 5mM MgCl₂], 0.1 mg/mL final protein concentration and 1.5 µM final concentration of 7-EFC) was added. The plate was pre-incubated at 37°C for 10 mins before 25 µL of NADPH regenerating system (1.7 mg/mL NADP⁺, 7.8 mg/mL G-6-P and 6U/mL G-6-P DH in 0.2% [v/v] NaHCO₃) was added to start the reaction. Fluorescent readings were taken every 1.5 mins for 30 minutes.

The rate of formation of fluorescent metabolite was plotted as a function of time and mean slopes in 8 solvent matched, uninhibited control wells on each plate were estimated to be 100% of activity. Slopes of wells containing potential inhibitors were expressed as a % activity of control wells and IC₅₀ values were determined using Xlfit software (version 5.2.2, IDBS, Guildford, Surrey, UK). Individual slope values were determined over 7 consecutive data-points along the time-course and IC₅₀ values were determined at each nominal time-point to see if the values increased or decreased with time. If there was a decrease over time, this was colour coded to show the trend, and how much it changed over the full timecourse (ie < or > 2-fold).

All experiments were repeated with 2 different batches of CYP2B6 and mean IC₅₀ values are presented.

Results

The compounds which were expected to be the most potent inhibitors of CYP2B6 from the literature with IC₅₀ or K_i values <10 µM were; Clopidogrel^{1,2}, Ticlopidine^{1,2,3,4}, 8-MOPS⁵, Montelukast¹, Tranylcypromine^{1,5,6}, Paroxetine¹ and Ketoconazole¹. Although the majority of these papers cite using HLM as the enzyme source for such studies, these same compounds were picked out as the most potent inhibitors in recombinant CYP2B6 (Table 1), although values tended to be lower than corresponding HLM values eg Fluvoxamine IC₅₀ value in HLM was 22 µM¹ and Troglitazone IC₅₀ value of 28 µM¹. Inhibition values have not been published in the literature for Mibefradil, Isoniazid, Tienilic acid, Cinacalcet and Miconazole (positive control, mean of n>10) due to CYP2B6 not being investigated alongside other CYPs as this has only become commercially available comparatively recently.

Only 8-MOPS, Chloramphenicol and N-Benzylphenobarbital caused a significant decrease in IC₅₀ value over a 30 minute incubation period. These compounds have previously been documented only as direct inhibitors of CYP2B6 with quite high IC₅₀ values; 8-MOPS (3-25 µM⁵), Chloramphenicol (>75 µM⁷) N-Benzylphenobarbital (62 µM⁸) but not as time/metabolism inhibitors of CYP2B6.

Ticlopidine^{3,4} and Clopidogrel² have been described as being time/metabolism dependent inhibitors of CYP2B6 in literature citations but mostly where HLM have been used as the enzyme source. The S-oxide metabolite which brings about this additional inhibitory element is formed by the action of enzymes other than CYP2B6^{2,9} so in a fluorimetric assay format this element is not detected.

References

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Table 1 IC₅₀ values (µM) for potential inhibitors of CYP2B6

Potential inhibitor	Solvent composition (v/v)			
	1.5% DMSO	2.5% DMSO	1.5% MeOH	2.5% MeOH
Furafylline	100.00	100.00	100.00	100.00
Fluvoxamine	3.97	4.25	7.70	3.80
Fluvoxamine (10-0.01)	7.33	7.48	10.00	10.00
Amiodarone	89.47	61.00	100.00	100.00
Mibefradil	96.00	100.00	100.00	47.50
Isoniazid	100.00	100.00	100.00	100.00
Clopidogrel	0.08	0.08	0.09	0.08
Clopidogrel (1-0.001)	0.06	0.06	0.04	0.03
Diltiazem	100.00	98.00	92.00	70.00
8-MOPS	4.15	3.43	4.25	2.57
Ticlopidine	0.10	0.08	0.11	0.08
Ticlopidine (1-0.001)	0.12	0.11	0.13	0.10
Montelukast	9.51	11.35*	10.65	11.65
Gemfibrozil	100.00	100.00	100.00	100.00
Fluoxetine	96.50	100.00	100.00	100.00
Troglitazone	6.25	7.65	100.00	6.10
Sulphafenazole	100.00	100.00	100.00	100.00
Tienilic acid	100.00	100.00	100.00	100.00
Tranylcypromine	2.72	3.18	5.28	3.07
Chloramphenicol	39.68	45.50	100.00	79.50
N-Benzylphenobarbital	45.13	50.95	85.75	43.66
Quinidine	100.00	100.00	100.00	100.00
Citalopram	100.00	100.00	100.00	100.00
Paroxetine	0.49	0.59	0.47	0.25
Cinacalcet	6.20	7.35	10.10	7.81
Ketoconazole	1.18	1.15	1.45	1.35
Ketoconazole (10-0.01)	1.73	2.23	3.25	2.67
TAO (USP)	100.00	100.00	100.00	100.00
TAO (Biotrend)	100.00	100.00	100.00	100.00
Erythromycin	100.00	100.00	100.00	100.00
Verapamil	100.00	100.00	100.00	100.00
Fluconazole	100.00	100.00	100.00	100.00
Miconazole (10-0.01)	0.036	0.028	0.054	0.029
Pearson rank coefficient	-	0.99	0.91	0.96

No colour denotes IC₅₀ value was <LLQ for the assay
 Yellow denotes IC₅₀ value showed some decrease along the time-course
 Orange denotes there was a consistent decrease in IC₅₀ value over the time-course this was < 2-fold
 Red denotes there was a consistent decrease in IC₅₀ value over the time-course this was > 2-fold
 * denotes slope of the curve did not fit the Hill equation and hence less emphasis should be placed on the end result

Conclusion

This fairly high throughput assay for identifying inhibitors of CYP2B6 with a fluorimetric endpoint is robust and reproducible. The assay correctly identifies potent inhibitors in addition to identifying if a compound's inhibitory effect increases over time, highlighting possible issues with dosage/safety.

8-MOPS and Chloramphenicol are suitable positive control inhibitors for highlighting time/metabolism dependency in this assay format.

Solvent choice and % composition had no effect on the inhibitory rank order of IC₅₀ values as demonstrated by the Pearson Rank Coefficient but 1.5% methanol (v/v) results were the most inconsistent.

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