

FLUOROMETRIC CYTOCHROMES P450 2C8, 2C9 AND 2C19 INHIBITION ASSAYS: TESTING THE ONE SUBSTRATE PARADIGM

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Abstract

We have developed microtiter plate-based, direct, fluorometric assays for the activities of several human drug metabolizing enzymes including CYP2C8, CYP2C9 and CYP2C19. Together, the CYP2C family participates in the metabolism of an estimated 15-20% of drugs and has been involved in several clinically significant drug interaction events. Several test compounds were examined for inhibition potency (IC_{50}) using dibenzyl fluorescein (DBF), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-methoxy-4-trifluoromethylcoumarin (MFC) and 7-ethoxy-3-cyanocoumarin (CEC) as substrates. Three substrates were examined for both CYP2C9 and CYP2C19, allowing intersubstrate comparison of IC_{50} s. For 10 compounds in which all three IC_{50} values were determined, 9 exhibited values within a 5-fold range. However,

ticlopidine exhibited an 8.6-fold range in IC_{50} values with CYP2C19. Additionally, both S- and R-warfarin exhibited activation of CYP2C9 with one of the substrates (MFC). Our data suggest that the use of single substrates is adequate in a CYP2C9 and CYP2C19 inhibitor screening regimen. Follow-up testing with an alternate substrate, particularly for CYP2C9, would be advisable before initiating substantial drug development efforts.

Introduction

In humans, CYP2C8, CYP2C9 and CYP2C19 are quantitatively important in the metabolism of drugs. As with all major drug metabolizing enzymes, co-administration of known substrates or inhibitors is a major source of adverse drug interaction events among drugs principally cleared by the CYP2C subfamily. The ability to predict drug interactions prior to clinic/market introduction is a current goal of the pharmaceutical industry.

In this report, we present an extension of the original high-throughput inhibition screen by Crespi *et al* (1997), focusing on the CYP2C enzymes. We describe the first application of this method to detect inhibitors of CYP2C8 and examine whether inhibitory effects are substrate independent among CYP2C9 and CYP2C19.

Material and Methods

Enzyme Assays. Incubations were conducted in a 200 μ l volume in 96 well microtiter plates (Catalog No. 3915, Corning Costar, Cambridge, MA) based on the method described on the Gentest Corporation website (www.gentest.com). A cofactor/serial dilution (C/SD) buffer was prepared that contained 2.6 mM NADP⁺, 6.6 mM glucose-6-phosphate ml, 0.8 U/ml glucose-6-phosphate dehydrogenase and 0.1 mg/mL microsomal protein prepared from baculovirus infected insect cells. To the first well in each row, 100 μ l of C/SD buffer that contained the upper concentration of inhibitor was added. In the second well, 50 μ l of that solution was added. In the second well and all remaining wells, 100 μ l of C/SD buffer that lacked test compound was added. The final concentration of the test substances in the first well varied between 50 μ M and 1000 μ M, depending on the solubility characteristics or potency of the inhibitor. Fifty microliters of the inhibitor solution from the second well in each row was dispensed into the third well and serially diluted 1:3 through the eighth well. Wells 9 and 10 contained no test substance and wells 11 and 12 were used as controls for background fluorescence (enzyme and substrate were added after the reaction was terminated). Addition of solutions to plates and serial dilutions were carried out by a liquid handling station (Multiprobe II, Packard Instruments). The plate was then pre-incubated at 37°C for 10 min, and the reaction initiated by the addition of 100 μ l of pre-warmed enzyme/

substrate (E/S) mix. The E/S mix contained buffer, cDNA-expressed P450 in insect cell microsomes, substrate and other components to give the final assay concentrations in a reaction volume of 200 μ l. Reactions were terminated after various times (see Tables 2-4) by addition of 75 μ l of a 4:1, acetonitrile:0.5 M Tris base solution, except for DBF where the reaction was terminated by the addition of 2 N sodium hydroxide. Fluorescence per well was measured using a BMG LabTechnologies, Inc. FLUOstar model 403 fluorescence plate reader (Durham, NC). In the case of DBF, fluorescence was measured after a minimum 2h delay necessary to reduce background fluorescence. Detection of the products of each assay was proportional with time and protein concentration. Data were exported and analyzed using an Excel spreadsheet. The IC_{50} values were calculated by linear interpolation.

Chemicals. The test substances and their supplier were as follows: cyclosporin A, quercetin, carbamazepine, Phenytoin, tranylcypromine, tolbutamide, ticlopidine, diclofenac, cimetidine, lansoprazole, indomethacin (Sigma-Aldrich); ketoconazole, sulfaphenazole S-warfarin, R-warfarin, S-mephenytoin, paclitaxel (Ultrafine Chemicals); Omeprazole was a generous gift of Dr. Joyce Goldstein, NIEHS. CEC was obtained from Molecular Probes (Eugene, OR). BFC, MFC and DBF were obtained from Gentest.

Table 1. Kinetic properties of the Substrate/Enzyme Pairs. Microsomal protein was standardized to 0.25 mg/mL.

Enzyme/Substrate pair	Km (μ M)	Vmax (min^{-1})
2C8/DBF	1.4	0.22
2C9/CEC	12	0.025
2C9/MFC	55	0.76
2C9/DBF	0.82	0.090
2C19/BFC	26	0.53
2C19/CEC	5.3	0.26
2C19/DBF	1.6	0.39

Table 2. Assay parameters – CYP2C8

Substrate	DBF
Concentration	1 μ M
pmol of enzyme/mL (0.2 mL per well)	20
Incubation time	30 min

Table 3. Assay parameters – CYP2C9

Substrate	CEC	MFC	DBF
Concentration	15 μ M	50 μ M	1 μ M
pmol of enzyme/mL (0.2 mL per well)	25	10	10
Incubation time	45 min	45 min	30 min

Table 4. Assay parameters – CYP2C19

Substrate	CEC	BFC	DBF
Concentration	6 μ M	25 μ M	2 μ M
pmol of enzyme/mL (0.2 mL per well)	5	25	5
Incubation time	30 min	45 min	30 min

Results

Assay Design

The assay design was similar to that originally reported by Crespi *et al.* (1997) in that IC_{50} values were determined using 8 inhibitor concentrations generated by serial 1:3 dilutions. However, several details were different in the present study. In particular, in order to better compare among substrates and to control for "futile" binding (Obach, 1997), the final microsomal protein concentration was standardized (to 0.25 mg/ml) by the addition of control microsomes. Apparent K_m values were measured under the standardized protein concentration and were found to be similar to previous determinations. All substrates were used at a concentration equal to the apparent K_m .

In addition, because of trend toward synthesizing compounds of limited aqueous solubility and the difficulties in solubilizing these compounds, control microsomes (0.1 mg/ml) were added to the serial dilution buffer.

Assay Variability

The mean and standard deviation for the replicate IC_{50} values were calculated for each determination (Table 5). For all CYP2C enzymes/substrate pairs, the within day CV was 0.09 and the between day CV was 0.25. This level of variability should be acceptable for screening applications.

Table 5. Assay variability – CV values

	CYP2C8	CYP2C9	CYP2C19	Grand Mean
Same substrates, within day	0.09	0.08	0.09	0.09
Same substrates, between day	0.12	0.42	0.21	0.25
Different substrates (n=3)	-	0.37	0.63	-

Table 6. Absolute and relative mean IC_{50} values (μ M)

CYP2C8	DBF										
Sulfaphenazole	156										
Carbamazepine	> 500										
Quercetin	1.9										
Phenytoin	> 100										
Paclitaxel	9.1										
Ketoconazole	4.4										
Cyclosporin	> 200										
Diclofenac	396										
CYP2C9	CEC	MFC	DBF	Overall	SD	CV	Range	Relative means			
Sulfaphenazole	0.30	0.51	0.25	0.35	0.13	0.4	2.0	0.86	1.43	0.72	
S-Warfarin	6.4	>200	7.1	-	-	-	>31	-	-	-	
R-Warfarin	13	>200	141	-	-	-	>15	-	-	-	
Tolbutamide	223	432	170	275	138	0.5	2.5	0.81	1.57	0.62	
Ketoconazole	4.5	5.1	2.9	4.2	1.16	0.3	1.8	1.09	1.22	0.69	
Tienilic Acid	0.14	0.50	0.34	0.33	0.18	0.5	3.5	0.44	1.53	1.02	
Diclofenac	2.8	2.5	2.1	2.5	0.34	0.1	1.3	1.12	1.03	0.85	
	mean						0.37	2.2	0.9	1.4	0.8
CYP2C19	CEC	BFC	DBF	Overall	SD	CV	Range	CEC	BFC	DBF	
Omeprazole	8.8	6.0	4.0	6.2	2.45	0.4	2.2	1.41	0.95	0.63	
Cimetidine	>200	47	81	109	80.26	0.7	>4.3	>1.83	0.43	0.75	
Ketoconazole	13	6.3	7.3	8.7	3.44	0.4	2.0	1.45	0.72	0.83	
S-Mephenytoin	>500	>500	301	-	n/a	n/a	>1.7	-	-	-	
Lansoprazole	1.3	1.4	0.36	1.0	0.59	0.6	3.9	1.29	1.36	0.35	
Tranylcypromine	4.0	5.3	1.9	3.7	1.73	0.5	2.8	1.07	1.43	0.51	
Indomethacin	> 200	> 200	> 200	> 200	n/a	n/a	-	-	-	-	
Ticlopidine	0.23	2.0	0.28	0.8	0.99	1.2	8.6	0.28	2.39	0.33	
	mean						0.63	3.9	1.1	1.2	0.6

Responses and IC_{50} Values for the Substrates

A summary of the IC_{50} values obtained for the test compounds is shown in Table 6. Except for S- and R-warfarin, a 1.3 to 8.6-fold range in mean IC_{50} values was found for a single compound with the 3 different substrates for CYP2C9 and CYP2C19. The mean range for CYP2C9 (except for warfarins) was 2.2 and for CYP2C19 it was 3.9. Most of the variability for CYP2C19 can be accounted for by the high IC_{50} for ticlopidine in the BFC assay. The 5-fold larger quantity of enzyme and longer incubation time may have resulted in inhibitor depletion resulting in higher IC_{50} values for the compounds which are also substrates.

Plots of representative experiments with CYP2C9 are shown in Figure 1. Dichotomous effects were observed for both S- and R-warfarin. Specifically, both isomers inhibited CYP2C9-CEC metabolism and both isomers activated CYP2C9-MFC metabolism. Assay interference was ruled out. These results may be consistent with the sigmoidal kinetics observed previously with certain CYP2C9 substrates (Korzekwa *et al.*, 1998). Interestingly, only the S isomer significantly inhibited CYP2C9-DBF metabolism. All other compounds tested with CYP2C9, as well as CYP2C19, exhibited concordance across the three substrates examined. Though 50% inhibition was found with paclitaxel at 9 μ M, this was near maximal and inhibition declined to ~10% at 200 μ M. This last effect may be due to the limited aqueous solubility of paclitaxel.

Summary and Conclusions

- The fluorometric CYP2C inhibition assays are reproducible and efficient.
- To our knowledge, DBF is the only available substrate to probe for inhibition of CYP2C8 in a high throughput regimen.
- Overall, there are substrate dependent differences in IC_{50} values with CYP2C9 and CYP2C19. The magnitude of these differences is less for CYP2C relative to CYP3A4.
- Our data suggest that the use of single substrates is adequate in a CYP2C9 and CYP2C19 inhibitor screening regimen. However, the results with warfarin isomers suggest that follow-up testing with an alternate substrate would be advisable with CYP2C9 before initiating substantial development efforts.

References

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Figure 1. Effects of selected test compounds on CYP2C9 catalytic activity using three substrates

