

CYTOCHROME P450 FLUOROMETRIC SUBSTRATES: IDENTIFICATION OF ISOFORM-SELECTIVE PROBES FOR RAT CYP2D2 AND HUMAN CYP3A4

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INTRODUCTION

Cytochrome P450 (CYP) catalytic activity assays that employ fluorometric substrates and are homogeneous ("mix and read") are desirable because they offer high sensitivity with rapid throughput. It would be of interest to identify fluorometric substrates that have the added advantage of selectivity for monitoring CYP isoform. We have tested a panel of nine fluorometric substrates with 29 human and rat cytochrome P450 isoform selectivity (247 enzyme/substrate pairs). Three of these substrates were found to be highly suitable for use as CYP isoform selective probes in rat and/or human liver microsomes.

Objectives:

- 1) To identify CYP isoform-selective fluorometric substrate probes for use with liver microsomes in homogeneous assays.
- 2) To characterize selected fluorometric substrate activity profile for human and rat CYP isoforms.

METHODS

Substrates:

Substrates and metabolites were obtained from BD Gentest or Ultrafine Chemicals. Metabolites were detected using the excitation and emission wavelengths shown in Table 1 below.

TABLE 1

Fluorometric substrates and their metabolite excitation and emission wavelengths	Substrate	Metabolite	Ex (nm)	Em (nm)
3-[2-(N,N-Diethylamino)ethyl]-7-hydroxy-4-methylcoumarin	AMMC	3-[2-(N,N-Diethylamino)ethyl]-7-hydroxy-4-methylcoumarin	390	460
5-[2-(N,N-Diethylamino)ethyl]-7-hydroxy-4-fluoromethylcoumarin	MeAMFC	5-[2-(N,N-Diethylamino)ethyl]-7-hydroxy-4-fluoromethylcoumarin	410	538
7-Methoxy-3-cyanocoumarin	CMC	7-hydroxy-3-cyanocoumarin	410	460
7-Ethoxy-3-cyanocoumarin	CEC	7-hydroxy-3-cyanocoumarin	410	460
7-Methoxy-4-fluoromethylcoumarin	MFC	7-hydroxy-4-fluoromethylcoumarin	410	538
7-Ethoxy-4-fluoromethylcoumarin	BFC	7-hydroxy-4-fluoromethylcoumarin	410	538
Dibenzofuran	DBF	Dibenzofuran	485	538
7-Benzoylquinoline	BQ	7-hydroxyquinoline	410	538
Resorufin benzyl ether	BzRes	Resorufin	530	590

Enzymes:

Microsomes were obtained from BD Gentest from baculovirus-infected insect cells (SUPERSOMES®) or from metabolically competent human B-lymphoblastoid cell lines that stably express rat CYP2E1 or rat CYP2A1. Liver microsomes from humans, Sprague-Dawley rats and Beagle dogs were obtained from BD Gentest. Liver microsomes from female Dark Agouti rats were a kind gift of Dr. Elizabeth Laurenzana (University of Arkansas for Medical Sciences, Little Rock, AR).

Incubations with cDNA-expressed enzymes and liver microsomes

Assays were conducted in 96-well microtiter plates (200 µl vol.). Buffer containing NADPH-regenerating system was added to wells. The plate was then warmed to 37°C and reaction initiated by the addition of pre-warmed enzyme/substrate mix. Reactions were terminated after 5-45 minutes by addition of 75 µl 80:20 acetonitrile:0.5 M Tris base or for DBF, 2 N NaOH. Additional parameters are provided in Tables 2-4 (bottom). Fluorescence was measured using a FLUOstar model 403 fluorescence plate reader (BMG Lab Technologies, Inc., Durham, NC) and the metabolite quantified by standard curves of the metabolite or external standard (3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin for AMMC only).

RESULTS

TABLE 2

Rat CYP Isoform	Substrate								
	AMMC	CMC	CEC	MFC	BFC	BQ	BzRes	DBF	DBF
CYP1A1	3.43	7.8	1.03	0.34	0.21	5.7	1.35	-	-
CYP1A2	2.21	1.38	1.32	-	0.41	2.3	0.62	-	-
CYP2A1	-	-	-	-	-	-	-	-	-
CYP2A2	0.24	0.45	0.08	0.26	0.030	2.8	0.82	-	-
CYP2B6	2.84	2.14	1.15	5.7	2.62	0.021	1.9	0.02	-
CYP2C8	1.53	0.55	-	0.13	0.085	-	-	-	-
CYP2C11	-	-	-	-	-	-	-	-	-
CYP2C12	-	-	-	0.24	-	-	1.5	-	-
CYP2C13	-	-	-	0.21	0.74	0.003	-	0.01	-
CYP2D2	0.01	-	-	0.08	0.34	0.159	5.3	0.1	-
CYP2E1	-	-	-	0.08	0.34	0.159	4.4	-	-
CYP3A1	-	-	-	0.01	0.08	-	-	-	-
CYP3A2	0.03	0.10	-	0.08	0.34	0.159	4.4	-	-
CYP3A4	0.01	0.08	-	1.33	0.027	8.4	0.06	-	-
CYP3A5	0.03	0.10	-	0.08	0.34	0.159	4.4	-	-
CYP3A7	-	-	-	-	-	-	-	-	-

Values, expressed as pmol product/min/pmol enzyme, represent the mean of duplicate determinations. Ranges were on average < 10% of the mean. Bold values indicate the highest catalytic activity within the panel.
- Dashes indicate activity was below the limit of detection which was considered as that corresponding to a signal to background ratio of approximately 1.5. These values and their substrates were 0.05 min⁻¹, AMMC: 0.05 min⁻¹, BFC: 0.008 min⁻¹, CMC: 0.008 min⁻¹, CEC: 0.003 min⁻¹, DBF: 0.05 min⁻¹, MFC: 1.0 min⁻¹, BQ: 0.008 min⁻¹, BzRes: -
+ Microsomes from human lymphoblastoid cells engineered to express the rat enzyme
- Concentration for all enzymes was 50 nM except for CYP2A1 (20 nM) and for CYP2E1 (87 nM)
- Concentration for all enzymes was 50 nM except for CYP1A1, which was 2.5 nM

TABLE 3

Human CYP Isoform	Substrate								
	AMMC	MeAMFC	CMC	CEC	MFC	BFC	BQ	BzRes	DBF
CYP1A1	0.15	3.25	46.2	3.01	0.68	2.32	3.2	0.457	-
CYP1A2	0.17	6.43	6.00	1.58	0.08	2.6	0.09	-	-
CYP1B1	0.10	0.29	0.65	0.68	0.20	0.01	-	1.55	-
CYP2A6	-	-	-	-	-	-	-	-	-
CYP2B6	-	-	0.52	0.59	10.1	-	-	-	0.020
CYP2C8	-	-	0.05	-	0.76	-	-	-	-
CYP2C9	-	-	0.11	0.35	1.10	-	-	-	-
CYP2C18	-	-	0.11	0.35	1.10	0.21	0.29	-	-
CYP2C19	-	-	0.11	0.35	1.10	0.21	0.29	-	-
CYP2D6	-	-	0.11	0.35	1.10	0.21	0.29	-	-
CYP2E1	-	-	0.11	0.35	1.10	0.21	0.29	-	-
CYP3A4	0.12	0.61	0.65	0.16	1.23	1.28	2.2	0.079	-
CYP3A5	-	-	-	-	-	-	-	0.41	0.011
CYP3A7	-	-	-	-	-	0.19	0.08	0.16	1.5
CYP3A8	-	-	-	-	-	-	-	-	-

Values, expressed as pmol product/min/pmol enzyme, represent the mean of duplicate determinations. Bold values indicate the highest catalytic activity within the panel.
- Dashes indicate activity was below the limit of detection which was considered as that corresponding to a signal to background ratio of approximately 1.5. These values and their substrates were 0.03 min⁻¹, AMMC: 0.07 min⁻¹, CMC: 0.07 min⁻¹, CEC: 0.007 min⁻¹, DBF: 0.08 min⁻¹, MFC: 1.5 min⁻¹, BQ: 0.008 min⁻¹, MeAMFC: 0.008 min⁻¹, BzRes: -
+ Except for CYP2D6, which was 7.5 nM
- Except for CYP1A1 which was 2.5 nM and CYP1A2 which was 0.5 nM
- Except for CYP1A1 and CYP2C19 which were 2.5 nM and CYP1A2 which was 0.5 nM
- Except for CYP3A4, CYP3A5, CYP3A7 which were 1.5 nM
- Except for CYP1A1 and CYP1B1 which were 5 nM

TABLE 4

Liver Microsomes Source	Substrate								
	AMMC	MeAMFC	CMC	CEC	MFC	BFC	BQ	BzRes	DBF
Human (n=29)	3.3 (2.2)	2.7 (0.3)	84 (38)	4.1 (1.4)	1.4 (0.2)	3.0 (1)	2.9 (1)	3.8 (1.9)	1.4 (0.3)
DA rat (n=1)	1.6 (4)	7.8 (23)	4.1 (4)	1.6 (1)	1.6 (1)	0.0 (0)	1.1 (2)	1.1 (2)	8.7 (10.8)
SD rat (n=1)	0.1 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Incubation time (min)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
Substrate concentration (µM)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
Enzyme concentration (pmol)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)

Values, expressed as pmol product/min/pmol enzyme, represent the mean of duplicate determinations.
- Values in parenthesis represent the range of duplicate determinations, except for DA rat where the values are SD of triplicate determinations.
+ Protein concentration for DA rat was 0.125 mg/ml, ND = Not Done

CONCLUSIONS

1. AMMC is a selective probe for CYP2D2 in rat liver microsomes.

Suggested conditions:

Buffer: 100 mM KPO4, pH 7.4
Substrate concentration: 25 µM
Protein concentration: 0.05 mg/mL
Incubation time: 30 min

2. BFC and BQ can be used as selective probes for CYP3A4 in human liver microsomes. There is a minor role of CYP1A2 in the metabolism of BFC.

Suggested conditions:

Buffer: 200 mM KPO4, pH 7.4
Substrate concentration: 50 to 100 µM (BFC) or 40 µM (BQ)
Protein concentration: 0.025 mg/mL (BFC) or 0.1 mg/mL (BQ)
Incubation time: 15 min (BFC) or 30 min (BQ)

DISCUSSION

AMMC for use as CYP2D2 probe in RLM.

Unlike humans, which express a single CYP2D enzyme (2D6), six CYP2D isoforms (CYP2D1/2/3/4/5/18) have been identified in rats. In the present study, cDNA-expressed CYP2D2, but not 13 other rat CYPs, catalyzed AMMC-demethylation. The selectivity of AMMC for CYP2D2 in rat liver microsomes was confirmed by demonstrating that AMMC-demethylase activity was 15-fold higher in SD rats than same activity in DA rats which lack functional CYP2D2. However, MFC demethylase activity, catalyzed primarily by CYP2C isoforms, was comparable between the two strains demonstrating that microsomes were metabolically competent. Thus, AMMC can be used as a selective probe for CYP2D2. The characterization of AMMC as a selective probe for CYP2D6 in HLM has been published elsewhere by us and others.

BFC for use as CYP3A4 probe in HLM.

In the enzyme panel, BFC was metabolized by CYP1A1, 1B1, 2C19, 3A4 and 3A7. Only CYP2C19 and CYP3A4 are expressed in adult liver, thus we pursued BFC as a selective probe for CYP3A4. Metabolism in a panel of microsomes from donors characterized for CYP isoform content demonstrated a significant correlation of BFC dealkylase activity with CYP3A4 (r = 0.95), but not CYP2C19 (r = 0.29) catalytic activity. When same analysis was conducted in the presence of 0.5 µM ketoconazole, a concentration that selectively inhibits CYP3A4, catalytic activity within the donor panel was decreased on average by 84% and a correlation with CYP3A4 was abolished. Instead, a significant correlation was found with CYP1A2 activity (r = 0.87). We compared Michaelis-Menten parameters of BFC metabolism in HLM with cDNA-expressed CYP1A2, 2C19 and 3A4. The K_M value for HLM was within 2.5-fold that of the cDNA-expressed enzymes. There was only slight evidence of saturation of CYP3A4 within the concentration range tested. To further characterize the role of CYP isoforms in the metabolism of BFC, chemical inhibition experiments in pooled HLM were performed. More than 90% inhibition was observed using ~ 0.5 µM ketoconazole. When α-naphthoflavone, a selective inhibitor of CYP1A2 and activator of CYP3A4 was tested, activation was clearly the predominant response, demonstrating a primary role for CYP3A4 in BFC metabolism. Quinidine and sulfaphenazole, selective inhibitors of CYP2D6 and CYP2C9, respectively, were without effect at 1-2 µM, concentrations at which they are considered selective. The low V_{MAX} values determined with cDNA-expressed CYP2C19 relative to CYP1A2 and CYP3A4 (10 to 100-fold less) suggests a negligible role for this enzyme in the metabolism of BFC in HLM. Together, these results suggest that CYP3A4 is the major hepatic enzyme (~85%) involved in the metabolism of BFC with CYP1A2 contributing to a much lesser degree.

BQ for use as CYP3A4 probe in HLM.

In the human enzyme panel, CYP3A4 was catalytically most active. In addition, CYP1A1, 1A2 and 3A5 were also found to metabolize BQ. Follow-up experiments were conducted similar to those with BFC. A correlation analysis in a panel of microsomes from donors characterized for CYP isoform content demonstrated a significant correlation of BQ dealkylase activity with CYP3A4 (r = 0.96) and CYP2B6 (r = 0.84) catalytic activities. We compared Michaelis-Menten parameters of BQ metabolism in HLM with cDNA-expressed CYP3A4. Both HLM and CYP3A4 displayed sigmoidal kinetics, a common observation for substrates of CYP3A4. To further characterize the role of CYP isoforms in the metabolism of BFC, chemical inhibition experiments in pooled HLM were performed. More than 90% inhibition was observed using ~ 0.5 µM ketoconazole, a concentration known to be selective for CYP3A4. When α-naphthoflavone, a selective inhibitor of CYP1A2 and activator of CYP3A4 was tested, activation was clearly the predominant response, demonstrating a primary role for CYP3A4 in BQ metabolism. Quinidine and sulfaphenazole, selective inhibitors of CYP2D6 and CYP2C9, respectively, were essentially without effect at 1-2 µM, concentrations at which they are considered selective. Because CYP1A1 is extrahepatic and CYP3A5 expression is low, these data suggest that CYP3A4 is the major hepatic enzyme involved in the metabolism of BQ (~85%) with CYP1A2 contributing to a much lesser degree.

TABLE 5

Substrate	Enzyme Source	K _M (nM)		V _{MAX} (pmol/min/mg)		Hill Coefficient (n)
		Human	Rat	Human	Rat	
BFC	HLM	39	1760	-	-	-
BFC	CYP3A4	99	96	-	-	-
BFC	CYP1A2	18	6.1	-	-	-
BFC	CYP2C19	28	0.53	-	-	-
BQ	HLM	63	6841	1.8	1.8	-
BQ	CYP3A4	22	26	-	-	-

Values expressed in units of pmol/min/mg (with LM) or pmol/min/pmol (with SUPERSOMES) - number of ligand binding sites bound by the substrate

TABLE 6

Human CYP Isoform	Catalytic Activity (pmol/min/mg)	Ketoconazole Inhibition (µM)			
		0	0.5	1.5	25
CYP1A2	0.60	0.67	-0.11	0.59	0.16
CYP2A4	0.14	-0.18	0.28	0.19	0.69
CYP2B6	0.88	0.93	0.84	0.31	0.19
CYP2C8	0.58	0.21	0.55	-0.24	-0.08
CYP2C9	0.37	0.50	0.30	-0.18	-0.03
CYP2C19	0.29	0.37	0.48	-0.31	-0.41
CYP2D6	-0.33	-0.11	-0.26	0.93	0.72
CYP2E1	0.28	0.34	0.24	0.22	-0.05
CYP3A4	0.95	0.88	0.98	-0.48	-0.47
CYP3A5	-0.22	0.46	-0.13	0.38	0.27
CYP3A7	0.76	0.83	0.83	-0.21	-0.16

* P < 0.05, ** P < 0.01

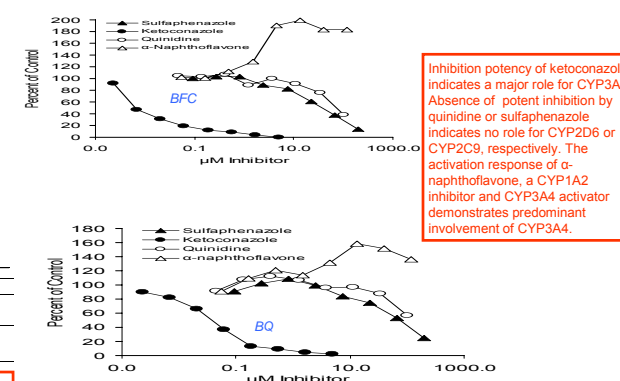


FIG 1. Effect of CYP isoform-selective chemical inhibitors on BFC (top) or BQ (bottom) dealkylase activity in human liver microsomes.