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Cytochrome P450 2D6.1 and cytochrome P450 2D6.10 differ in catalytic activity for multiple substrates

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CYP2D6 is involved in the metabolism of several classes of drugs, including tricyclic antidepressants, selective serotonin reuptake inhibitors and various amphetamines. *CYP2D6*10* is an allelic variant, producing an enzyme with Pro[34]Ser and Ser[486]Thr amino acid substitutions. Approximately 75% of Asians possess the *10 allele. We sought to further characterize CYP2D6.10 catalytically *in vitro* in a baculovirus expression system using various substrates and inhibitors, in comparison to CYP2D6.1 (wild-type). Using dextromethorphan (DEX), *P*-methoxyamphetamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and (±)3,4-methylenedioxymethamphetamine (MDMA), the ratios of intrinsic clearance (V_{max}/K_m) of *1 to *10 were 50, 34, 22 and 123, respectively. The CYP2D6 substrates amitriptyline, and (+) and (−) methamphetamine (MAMP) are both *p*-hydroxylated and *N*-demethylated (NDM). The intrinsic clearance *1/*10 ratios were 42, 30 and 67 for the *p*-hydroxylation; and 60, 120 and 157 for the NDM, respectively, illustrating chemical pathway and enantiomeric selectivity for MAMP. It was apparent that (+) and (−) MAMP NDM and MDMA demethylenation were most significantly different in CYP2D6.10. Using DEX as the substrate, the ratios of K_i(*10)/K_i(*1) for inhibitors were: budipine (1.3), sparteine (1.6), debrisoquine (8.1), fluoxetine (16), norfluoxetine (30), paroxetine (14), MDMA (21) and MMDA-2 (7.1), indicating that CYP2D6.10 shows drug-specific altered susceptibility to inhibition. Taken together, these data suggest that *CYP2D6*10/*10* individuals may be expected to require different drug doses; and show altered susceptibility to toxicity, interaction risk and, in the case of the amphetamines, drug dependence and toxicity compared to *CYP2D6*1/*1* individuals.

Keywords: CYP2D6, *10 allele, drug metabolism, amphetamines

Introduction

Cytochrome P450s (CYPs) are the major Phase I biotransformation enzymes, responsible for maintaining homeostasis in metabolizing both endogenous substances and exogenous xenobiotics (Parkinson, 1996). A major cause of interindividual and intergroup variation in drug effect is genetic variation of drug metabolism (Kalow, 1991). CYP2D6 is a polymorphic enzyme, resulting in a bimodal distribution of extensive and poor metabolizers of CYP2D6 substrates in Caucasian populations (Maghoub *et al.*, 1977; Eichelbaum *et al.*, 1979; Bertilsson *et al.*, 1992). 5–10% of Caucasians are classified as CYP2D6 poor metabolizers in contrast to only 1% of Asians (Alvan *et al.*, 1990; Bertilsson *et al.*, 1992). Despite the very low prevalence of CYP2D6 poor metabolizers in Asians, this group displays lower mean CYP2D6 activity, as represented by a right-shift in the metabolic ratio for several CYP2D6 substrates, including debrisoquine, codeine and sparteine (Lou *et al.*, 1987; Yue *et al.*, 1989; Bertilsson *et al.*, 1992; Yokota *et al.*, 1993). This lower overall CYP2D6 activity has been attributed to the high frequency of the CYP2D6.10 enzyme, possessing Pro[34]Ser and Ser[486]Thr amino acid mutations, present in approximately 75% of Asians (Wang *et al.*, 1993; Johansson *et al.*, 1994). Recent clinical studies have demonstrated increased plasma concentrations and areas under concentration–time curves in *CYP2D6*10/*1* and *CYP2D6*10/*10* individuals versus *CYP2D6*1/*1* individuals for a number of

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CYP2D6 substrates, including nortriptyline, venlafaxine, haloperidol, codeine and dextromethorphan (Yue *et al.*, 1998; Caraco *et al.*, 1999; Fukuda *et al.*, 1999; Mihara *et al.*, 1999; Tateishi *et al.*, 1999; Yue *et al.*, 1999). In a previous in-vitro study characterizing CYP2D6.10, Johansson *et al.* demonstrated a significant reduction in protein levels and bufuralol 1′-hydroxylation in COS-1 cells expressing both Pro^{34}-Ser and Ser^{486}Thr mutations (Johansson *et al.*, 1994). Detailed kinetic analyses, however, were not performed and only one substrate, bufuralol, was examined (Johansson *et al.*, 1994). A recent study by Fukuda *et al.* examined two substrates, venlafaxine and bufuralol, and found reduced affinity of CYP2D6.10 for these compounds (Fukuda *et al.*, 2000). The focus of this investigation therefore was to examine the kinetic interactions of a panel of diverse CYP2D6 substrates and inhibitors with CYP2D6.10 *in vitro*. Differential metabolism and/or inhibition by these compounds in CYP2D6.10 will have implications on drug dosing, toxicity and the likelihood of drug–drug interactions in those with this variant enzyme. Ethnic variability in these kinetic effects may also be predicted, allowing for ethnic-specific pharmacotherapy.

Materials and methods

CHEMICALS

Dextromethorphan hydrobromide, dextrorphan tartrate, 3-hydroxymorphinan hydrochloride and 3-methoxymorphinan hydrobromide were provided by Hoffmann-LaRoche Inc. (Nutley, NJ, USA). Butorphanol tartrate, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), amitriptyline hydrochloride, nortriptyline hydrochloride, desipramine hydrochloride, sparteine, tryptamine, β-NADPH sodium salt and superoxide dismutase were purchased from Sigma Co. (St Louis, MO, USA). (±)Norfluoxetine maleate and (±)fluoxetine were obtained from Eli Lilly Co. (Indianapolis, IN, USA). Debrisoquine sulfate was purchased from Research Biochemicals International (Natick, MA, USA). PTP was purchased from Toronto Research Chemicals (Ontario, Canada). (±)3,4-Dihydroxymethamphetamine was kindly provided by Dr Arthur Cho. All other amphetamine analogues were provided by the National Institute on Drug Dependence, USA. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethyl acetate, ethyl ether, hexane and dichloromethane were all HPLC-grade and purchased from Caledon Laboratory Chemicals (Georgetown, ON, Canada). Potassium phosphate monobasic, heptane sulfonic acid, dibasic sodium carbonate and sodium bicarbonate were all ACS-grade.

CDNA-EXPRESSING MICROSOMES

cDNA-expressing microsomes were purchased from GenTest Co. (Woburn, MA, USA). CYP2D6.1 and CYP2D6.10 were each coexpressed with human cytochrome P450 oxidoreductase in BTI-TH-5B1-4 insect cells using a baculovirus expression vector. Both expression systems contain similar reductase to P450 ratios (0.9 : 1.0).

HPLC

A Hewlett-Packard 1100 isocratic pump, with a 1050 autosampler, 1050 UV detector and a HP339611 integrator were used for analyses. For MPTP analyses, a complete 100 HP 1100 series system with Chemstation was utilized.

SUBSTRATE ASSAYS

Incubations were carried out in 0.1 M KH_2PO_4 buffer (pH 7.4) at 37 °C under continuous shaking. Preliminary studies were performed, using dextromethorphan (DEX) as the substrate, to determine incubation conditions under which dextrorphan (DOR) formation was linear with respect to time and CYP concentration. Incubation conditions were chosen for the substrates investigated following the establishment of linear conditions of product formation. Substrate, buffer and 1 mM NADPH were mixed in a final volume of 200 µl (except for MPTP where the final volume was 500 µl), and the reactions were initiated by the addition of enzyme. Total protein concentration was normalized in each assay, except *p*-methoxyamphetamine (PMA), by the addition of control insect cell microsomes provided by GenTest Co. For (±)3,4-methylenedioxymethamphetamine (MDMA) studies, 30 U of superoxide dismutase were added to minimize conversion of dihydroxymethamphetamine to its *O*-quinone. Initial experiments to determine enzymatically formed peaks indicated a small production, of approximately 15%, of amphetamine from higher concentrations of methamphetamine (MAMP) (above 35.3 µM), even when no enzyme was present, due to the apparent spontaneous chemical *N*-demethylation of MAMP. This 'background' amphetamine production was linear with the amount of MAMP incubated. In addition, similar amounts of amphetamine were produced in the absence of enzyme as in the presence of enzyme (without NADPH). Experiments were thus performed

to measure this background amphetamine production, where all conditions and drug concentrations were identical, except that no enzyme was added. Enzymatically derived amphetamine for both CYP2D6.1 and CYP2D6.10 was then calculated by subtracting the amount of amphetamine formed in the presence of enzyme by the amount formed in the absence of enzyme. Substrate concentrations, enzyme amounts and incubation times used for each assay are summarized in Table 1.

EXTRACTION

Dextromethorphan

Extraction procedure was essentially that of Chen *et al.* (1990). After incubation, samples were placed on ice and 100 µl of sodium bicarbonate buffer (pH 9.9) was added. The reaction was terminated by the addition of 1 ml of hexane : ether (4 : 1). Samples were vortexed and centrifuged for 10 min each, and back-extracted with 100 µl of 0.01 N hydrochloric acid. Organic solvent was then aspirated, and 30 µl of the remaining sample was injected into the HPLC [C_5 Waters Spherisorb column; flow rate = 1 ml/min; UV = 200 nm; 10 mM KH_2PO_4 buffer containing 1 mM heptane sulphonic acid and acetonitrile (73:27, pH 3.8)]. For CYP2D6.10 studies, norfluoxetine was used as the internal standard; for CYP2D6.1 and all inhibition studies, butorphanol was used as the internal standard.

PMA

Extraction protocol was modified from that of Li *et al.* (1997). After incubation, samples were placed on ice and 300 µl of sodium bicarbonate buffer (pH 9.9) was added. 3 ml of ethyl acetate was added in the presence of approximately 0.2 g NaCl crystals. Samples were shaken horizontally for 30 min, centrifuged for 10 min, and back-extracted with 200 µl of 0.01 N HCl. Organic phase was aspirated, and 50 µl was injected into the HPLC [C_6 Waters Spherisorb column; flow rate = 1 ml/min; UV = 214 nm; 10 mM KH_2PO_4 buffer and acetonitrile (80:20, pH 6.0)]. 2-Methoxyamphetamine was used as the internal standard.

(±) and (−) Methamphetamine

Extraction and HPLC conditions were identical to those for PMA. 3-Methoxyamphetamine was used as the internal standard.

Amitriptyline

Extraction and HPLC conditions were identical to those for DEX. Desipramine was used as the internal standard.

MPTP

The reaction was terminated by the addition of 4 ml of dichloromethane. Samples were vortexed for 20 min and then centrifuged. The organic phase was then evaporated to dryness under nitrogen and the residue was redissolved in 200 µl of mobile phase. Fifty µl was then injected into the HPLC [CSC-Spherisorb-phenyl column; flow rate = 1 ml/min; UV = 244 nm; 100 mM KH_2PO_4 buffer and acetonitrile (88 : 12; pH 3.0)]. Diethyltryptamine was used as the internal standard.

Table 1. Experimental parameters used for substrates investigated

Substrate	*Substrate concentration range (µM)*	*CYP amount used (pmol)*		*Incubation time (min)*	
		CYP2D6.1	*CYP2D6.10*	*CYP2D6.1*	*CYP2D6.10*
Dextromethorphan (DEX)	0.5–312.5	1.5	25.0	15	30
p-methoxyamphetamine (PMA)	2.5–1000	2.5	25.0	20	40
(+)/(−) Methamphetamine (MAMP)	4.2–293[a]	20.0	50.0	45	60
Amitriptyline (AMI)	1.25–400	2.5	20.0	20	30
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	3.9–500	1.3	25.0	10	30
(±)3,4-methylenedioxy-methamphetamine (MDMA)	0.5–173	1.5	15	10	25

[a]For CYP2D6.10, three to five concentrations points ranging from 35 to 1000 µM were used due to the high cost of the enzyme and peak interference at higher substrate concentrations.

MDMA

The reactions were stopped by the addition of 15 µl of 70% perchloric acid. Samples were vortexed for 20 min and centrifuged to pellet the protein. Seventy µl of the top layer was then injected into the HPLC [Hewlett-Packard Spherisorb ODS-2 column; flow rate = 1 ml/min; UV = 205 nm; 10 mM KH_2PO_4 buffer containing 1 mM heptane sulphonic acid and acetonitrile (92:8; pH 2.8)]. MDMA could not be detected at the conditions used. Tyramine was used as the internal standard. Variation for all assays was under 14%.

INHIBITION ASSAYS

DEX was incubated at final concentrations of 0.5 K_m, K_m, and 2 K_m for CYP2D6.1 and CYP2D6.10. Therefore, final DEX concentrations used were 0.5 µM, 1 µM and 2 µM for CYP2D6.1; and 2 µM, 4 µM, and 8 µM for CYP2D6.10. Due to the high expense of the enzymes, 2-line K_is (at K_m and *2* K_m of DEX) were determined for budipine, MMDA-2 and MDMA. DOR production was monitored in the presence and absence of four different concentrations of each chemical inhibitor (50 µl). Inhibitor concentrations were chosen based on initial screening experiments in which inhibitor concentrations of K_i and 25 K_i, based on previously published data, were incubated with 1 µM of DEX. After the addition of inhibitor to DEX, 0.1 M sodium phosphate buffer and 50 µl of NADPH (1 mM) were added. Incubation conditions are listed in Table 1.

DATA ANALYSIS

Kinetic parameters were calculated using Enzfitter computer software, version 1.05 (Elsevier Biosoft Inc., Amsterdam, The Netherlands) when the involvement of one enzyme was indicated. In the case of two-enzyme kinetics, Graphpad Prism software version 2.01 (Graphpad Software Inc., San Diego, CA, USA) was used. Dixon and Cornish–Bowden plots were generated using Microsoft Excel 97.

STATISTICAL ANALYSIS

A one-way ANOVA was used to determine the coefficient of variation based on replicates of K_i, $K_m$and V_{max} values for eight substrate/inhibitor–enzyme combinations. The log ratios of K_i in **10* to K_i in **1* were sorted and compared using the Duncan New Multiple Range Test ($P < 0.05$), allowing determination of significant pairwise differences.

Results

Kinetics in CYP2D6.1 and CYP2D6.10

K_m, V_{max} and intrinsic clearance (V_{max}/K_m) values, determined in CYP2D6.1- and CYP2D6.10- expressing microsomes, are summarized in Table 2. Due to the high expense of these enzymes, replicate determinations were only conducted for selected substrates. As shown in Table 2, large, substrate-dependent differences were observed between kinetic constants obtained with CYP2D6.10 compared to those obtained with CYP2D6.1.

DEX, a selective CYP2D6 probe substrate which is O-demethylated to DOR, displayed average K_m values of 0.8 and 5.0 µM in CYP2D6.1 and CYP2D6.10 (Figs 1a,b), respectively; and an approximate 50-fold attenuated intrinsic clearance in CYP2D6.10.

PMA, a hallucinogenic central nervous system stimulant, is *O*-demethylated to its less active metabolite, *p*-hydroxyamphetamine. Similar to DEX, PMA demonstrated both reduced metabolism and affinity with CYP2D6.10 versus CYP2D6.1. K_m values of 37 and 78 µM were determined in CYP2D6.1 and CYP2D6.10, respectively, and a 34-fold reduced in-vitro intrinsic clearance was observed with CYP2D6.10.

MAMP, a potent CNS stimulant, is both *N*-demethylated and *p*-hydroxylated to the less active metabolites, amphetamine and *p*-hydroxymethamphetamine, respectively (Cho & Kumagai, 1994), and exists as both (+) and (−) enantiomers. With CYP2D6.1, *N*-demethylation of both (+) and (−)MAMP was more efficiently catalysed than the *p*-hydroxylation reaction, as demonstrated by the approximately three-fold higher intrinsic clearance values observed with *N*-demethylation (Table 2). K_m values of 12 and 285 µM were obtained with (+)MAMP *N*-demethylation (Fig. 1c); while K_m values of 28 and 300 µM were determined with (−)MAMP *N*-demethylation, in CYP2D6.1 and CYP2D6.10, respectively. For *p*-hydroxylation, K_m values of 23 and 97 µM were obtained with (+)MAMP; while K_m values of 52 and 385 µM were obtained with (−)MAMP in CYP2D6.1 and CYP2D6.10, respectively. Therefore, with (+)MAMP, intrinsic clearance was reduced by 120-fold for the *N*-demethylation reaction in CYP2D6.10, but only 30-fold for the *p*-hydroxylation reaction. With (−)MAMP, a more pronounced attenuation was again seen with *N*-demethylation versus *p*-hydroxylation in CYP2D6.10, at 157-fold and 67-fold, respectively compared to CYP2D6.1.

Amitriptyline (AMI), similar to MAMP, is both *N*-demethylated and *p*-hydroxylated, to nortriptyline and E-10-hydroxyamitriptyline, respectively. How-

Table 2. Summary of kinetic properties of various CYP2D6 substrates in CYP2D6.1- and CYP2D6.10-expressing microsomes from insect cells

Substrate	K_m *(μM)*	V_{max} *(pmol/pmol 2D6/min)*	*Intrinsic clearance* (V_{max}/K_m) *(μl/pmol 2D6/min)*	*Ratio of intrinsic clearance*[a]
Dextromethorphan	0.8 (0.9, 0.8)	2.1 (2.0, 2.1)	2.6 (2.0, 2.8)	50
	5.0 (4.6, 5.3)	**0.26 (0.23, 0.28)**	**0.052 (0.050, 0.053)**	
p-methoxy-amphetamine	37 (37, 36)	14 (15, 13)	0.37 (0.40, 0.36)	34
	78 (70, 85)	**0.86 (0.79, 0.92)**	**0.011 (0.011, 0.011)**	
(+)methamphetamine: *N*-demethylation	12 (13, 12, 11)	0.44 (0.46, 0.41, 0.44)	0.036 (0.037, 0.037, 0.035)	120
	285	**0.081**	**0.00030**	
(+)methamphetamine: *p*-hydroxylation	23 (23, 23)	0.25 (0.23, 0.27)	0.012 (0.012, 0.011)	30
	97	**0.040**	**0.00041**	
(−)methamphetamine: *N*-demethylation	28	0.31	0.011	157
	300	**0.020**	**7.0×10^{-5}**	
(−)methamphetamine: *p*-hydroxylation	52	0.22	0.0040	67
	385	**0.023**	**6.0×10^{-5}**	
MPTP	43	11	0.26	22
	103	**1.2**	**0.012**	
Amitriptyline: *N*-demethylation	9.2	2.2	0.24	60
	42	**0.17**	**0.0040**	
Amitriptyline: *p*-hydroxylation	1.8	1.6	0.89	42
	5.8	**0.12**	**0.021**	
MDMA	1.3 (1.6, 1.0)	4.0 (5.0, 2.9)	3.1 (3.2, 2.9)	123
	21 (16, 26)	**0.53 (0.47, 0.60)**	**0.025 (0.030, 0.023)**	

[a](V_{max}CYP2D6.1/K_mCYP2D6.1)/(V_{max}/CYP2D6.10/K_mCYP2D6.10). Results are for in CYP2D6.1 and **CYP2D6.10**.

ever, unlike MAMP, both the *N*-demethylation and *p*-hydroxylation of AMI were affected similarly in CYP2D6.10. K_m values of 9.2 and 42 μM were obtained for *N*-demethylation; while K_m values of 1.8 and 5.8 μM were obtained for *p*-hydroxylation in CYP2D6.1 and CYP2D6.10, respectively. Intrinsic clearances were reduced by 60- and 42- fold for AMI *N*-demethylation and *p*-hydroxylation in CYP 2D6.10, respectively compared to CYP2D6.1.

MPTP is a neurotoxin which is *N*-demethylated to 4-phenyl-1,2,3,6-tetrahydropyridine (PTP). MPTP metabolism in CYP2D6.10 was the least different from CYP2D6.1, although intrinsic clearance was still reduced by an approximately 20-fold in CYP2D6.10. K_m values of 43 and 103 μM were determined in CYP2D6.1 and CYP2D6.10, respectively.

In contrast to MPTP, MDMA demethylenation (to 3,4-dihydroxymethamphetamine) in CYP2D6.10 was among the compounds most different from CYP2D6.1. K_m values of 1.3 and 21 μM were determined in CYP2D6.1 and CYP2D6.10, respectively, for MDMA demethylenation. Accordingly, intrinsic clearance values were substantially reduced in CYP2D6.10, demonstrating approximately 120-fold less activity than CYP2D6.1.

Differences between substrates

The decreases in intrinsic clearance seen with all of the substrates investigated were a result of both decreases in substrate affinity (K_m) and reaction velocity (V_{max}). These effects, however, were not uniform across the substrates. To assess variation between substrates and chemical pathways, we determined statistically significant differences in the ratios of K_m and intrinsic clearance values obtained in CYP2D6.10, compared to those obtained in CYP2D6.1. The affinities of (+) and (−)MAMP *N*-demethylation (NDM) and MDMA demethylenation for CYP2D6.10 were the most attenuated, relative to

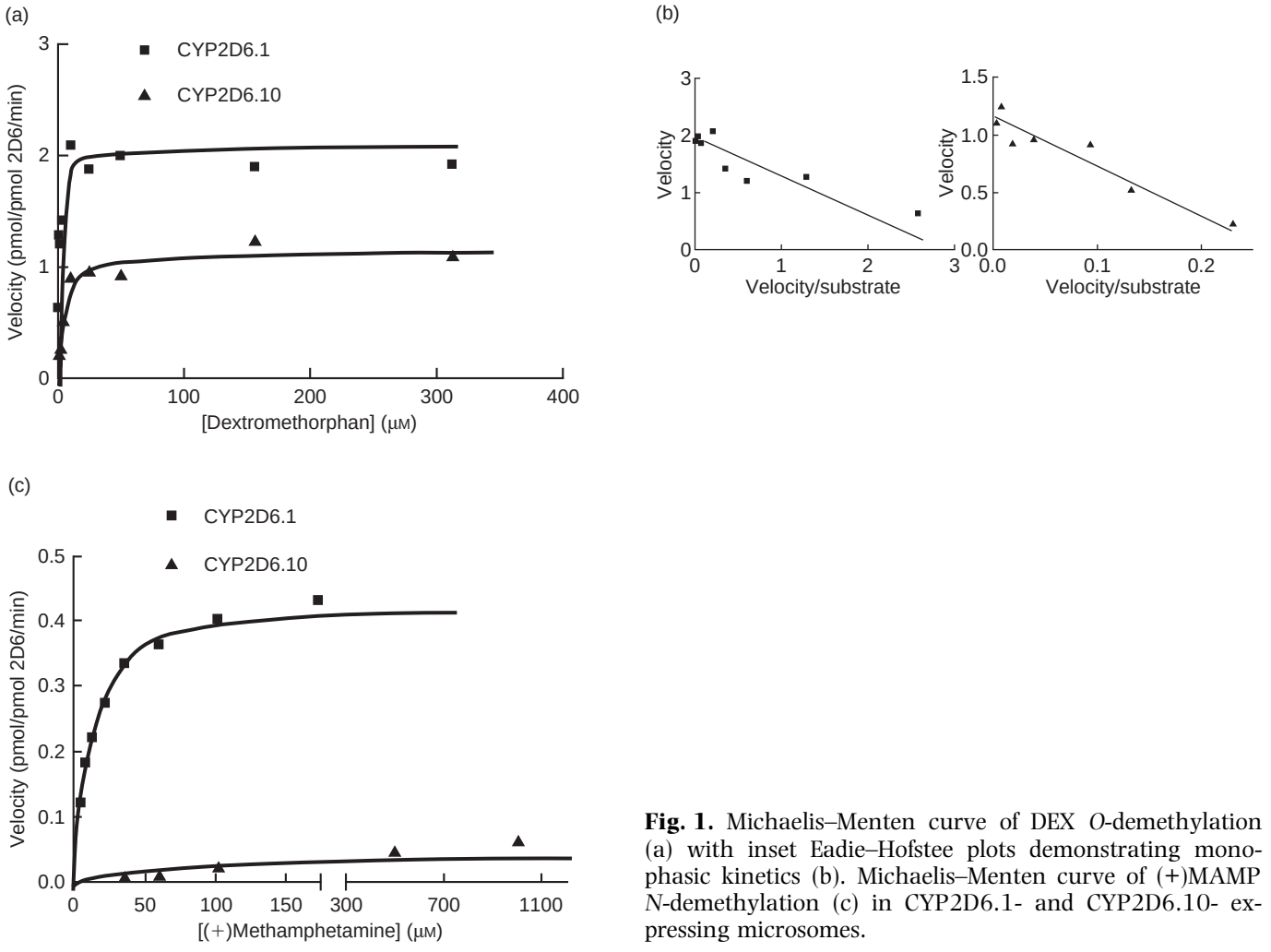

Fig. 1. Michaelis–Menten curve of DEX *O*-demethylation (a) with inset Eadie–Hofstee plots demonstrating monophasic kinetics (b). Michaelis–Menten curve of (+)MAMP *N*-demethylation (c) in CYP2D6.1- and CYP2D6.10- expressing microsomes.

CYP2D6.1, demonstrating 24-fold, 11-fold and 16-fold higher K_m values, respectively. Conversely, the affinities of *p*-methoxyamphetamine and MPTP for CYP2D6.10 were the most similar to CYP2D6.1, both displaying only approximately two-fold increases in K_m in CYP2D6.10. Chemical pathway selectivity was also evident, with the K_m for (+)MAMP NDM being significantly more affected in CYP2D6.10 than (+)MAMP *p*-hydroxylation.

With intrinsic clearance (+) and (−)MAMP *N*-demethylation (NDM), MDMA demethylenation and MPTP NDM displayed significantly different ratios from the greatest number of other substrates, with (−)MAMP NDM (+)MAMP NDM and MDMA being significantly smaller than several other substrates. Conversely, the log intrinsic clearance ratio (**10/*1*) for MPTP (+)MAMP *p*-hydroxylation and PMA were significantly higher than those for several other substrates.

There were also significant differences between intrinsic clearance ratios (**10/*1*) between the enantiomers of MAMP, with the ratio for (−)MAMP *p*-hydroxylation being significantly lower than that for (+)MAMP. Differences between the metabolic pathways of (+) and (−)MAMP were also noted, with the NDM of both (+) and (−)MAMP being significantly lower than for the *p*-hydroxylation pathway, with the effects being more predominant with (+)MAMP. No significant differences were seen amitriptyline NDM and its *p*-hydroxylation.

Inhibition of CYP2D6.1 and CYP2D6.10

In addition to substrates, the interactions of a number of inhibitors of varying drug classes with CYP2D6.1 and CYP2D6.10 were studied. DEX was chosen as the probe CYP2D6 substrate because of its high selectivity and specificity for CYP2D6. The inhibitors were chosen in an effort to select compounds from different drug classes to allow for assessment of potential differences between drug classes and chemical structures. Norfluoxetine, fluoxetine and paroxetine are all selective serotonin reuptake inhibitors (SSRIs). MMDA-2 and MDMA are both methylenedioxy-substituted amphetamines.

Sparteine and debrisoquine are not from the same drug class but, along with dextromethorphan, are commonly used CYP2D6 probe substrates used for phenotyping. Therefore, inhibition studies with these compounds revealed potential differences in their metabolism by CYP2D6.10. Finally, budipine is a highly selective probe CYP2D6 inhibitor, which was used as a positive control for CYP2D6 inhibition. Average K_i values, with individual determinations in parentheses, for all the compounds examined are listed in Table 3. All compounds demonstrated competitive inhibition of CYP2D6.1 and CYP2D6.10, except paroxetine, which displayed mixed inhibition in CYP2D6.1. All inhibitors displayed greater K_i values in CYP2D6.10 than in CYP2D6.1, though to varying degrees. The ratios of K_i values obtained in CYP2D6.10 compared to those in CYP2D6.1 varied from 1.3 and 1.6 for budipine and sparteine, respectively, showing little difference between the two alleles, to 20- and 30-fold for MDMA and norfluoxetine, respectively, demonstrating significant differences between CYP2D6.1 and CYP2D6.10. This 20-fold difference in K_i values for MDMA was very similar to the 16-fold difference obtained with K_m, providing validation of the significant attenuation of MDMA affinity for CYP2D6.10. It was also demonstrated that debrisoquine displayed a significantly greater K_i ratio (*10/*1) (8.3) than did sparteine (1.6), illustrating differential interactions of the two compounds with CYP2D6.10. Dixon and Cornish–Bowden plots for sparteine in CYP2D6.1 and CYP2D6.10, indicating competitive inhibition, are illustrated in Fig. 2.

Discussion

With all the substrates examined, CYP2D6.10 exhibited decreased metabolism and reduced affinity, as compared to CYP2D6.1 (Table 2). These effects manifested as reductions in in-vitro intrinsic clearances of all the substrates. The reduced substrate affinity observed here is in line with a recent study demonstrating an approximate five-fold reduction in K_m values for bufuralol and venlafaxine in cDNA-expressed CYP2D6.10 versus CYP2D6.1 (Fukuda *et al.*, 2000). In the present study, several different substrates were examined enabling some distinctions between substrates to be made. It was found that the K_m ratios (*10/*1) for (+)MAMP NDM and MDMA demethylenation were significantly lower than seven and five other substrates, respectively. In contrast to MAMP and MDMA, PMA and MPTP were among the least different in both drug affinity and intrinsic clearance in CYP2D6.10 versus CYP2D6.1. Differential effects on affinity for different CYP2D6 substrates have been previously observed with CYP2D6.17, where K_ms for codeine and bufuralol were approximately five-fold versus 10- fold greater, respectively, compared to CYP2D6.1 (Oscarson *et al.*, 1997). CYP2D6.10 has single amino acid mutations including one that is also present in CYP2D6.17 ($Ser^{486}Thr$) and has been implicated in enzyme–substrate interactions (Gotoh, 1992), which may underlie the differences in substrate affinity that were observed. The particularly profound effects with MAMP and MDMA in CYP2D6.10 suggest that certain key chemical components on these molecules

Table 3. Summary of inhibition of dextromethorphan metabolism in CYP2D6.1- and CYP2D6.10-expressing microsomes

Inhibitor	K_i *CYP2D6.1 (μM)*	K_i *CYP2D6.10 (μM)*	*Ratio of (K_iCYP2D6.10)/ (K_iCYP2D6.1)*
Sparteine	9.7 (8.0, 14, 7.5)	16 (15, 15, 17)	1.6
Debrisoquine	1.8 (2.0, 1.5)	15	8.3
Budipine	0.3	0.4 (0.5, 0.4)	1.3
MDMA	0.4	8.2	21
MMDA-2	0.4 (0.3, 0.4)	2.5	7.1
Fluoxetine	0.4 (0.6, 0.3)	6.8	17
Norfluoxetine	0.6 (0.7, 0.4)	17	31
Paroxetine	0.2 (0.2, 0.3)	3.3	13

K_i, inhibition constant. K_i values were determined in triplicate on different days for one inhibitor (sparteine) in both CYP2D6.1- and CYP2D6.10-expressing microsomes. For most of the other inhibitors, K_is were determined in duplicate in CYP2D6.1 and singly in CYP2D6.10 because of the high expense of the enzymes.

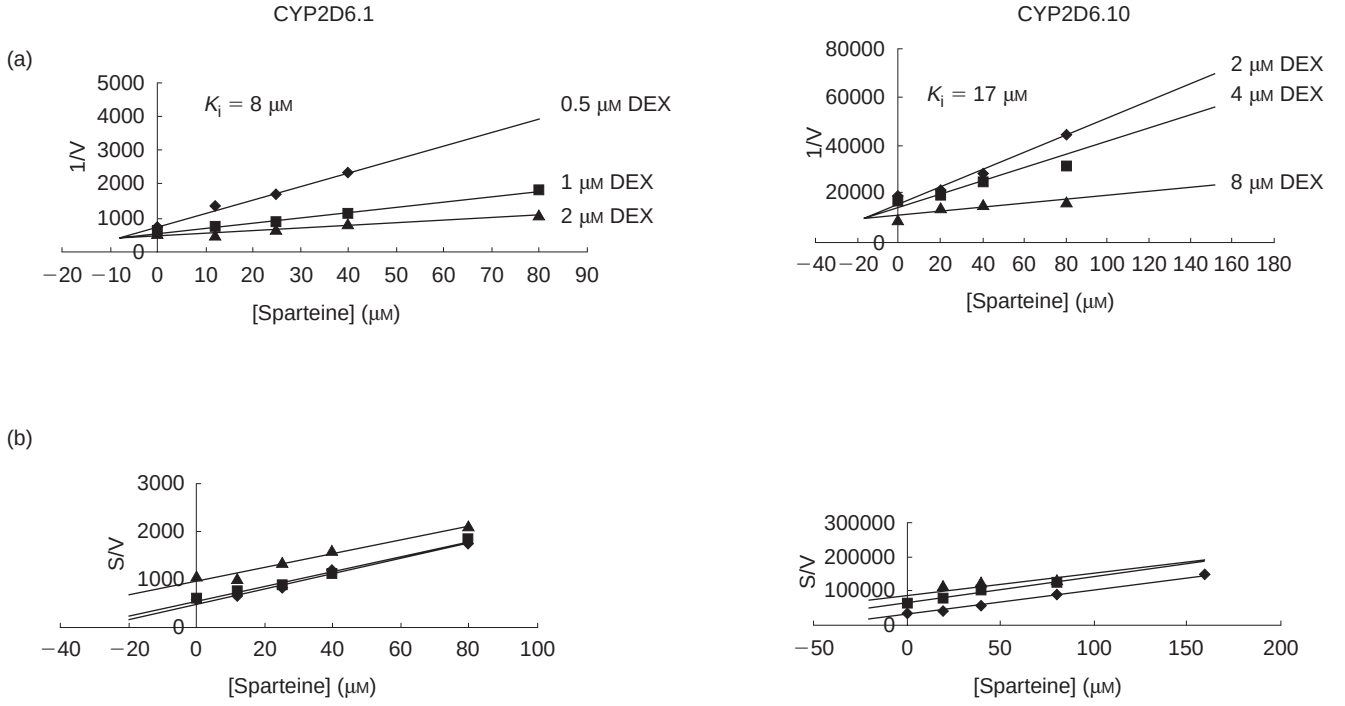

Fig. 2. Dixon (a) and Cornish–Bowden plots (b) illustrating competitive inhibition of DEX with sparteine in CYP2D6.1- (left) and CYP2D6.10- (right) expressing microsomes. V, velocity; S, substrate.

may be especially important in constraining their interactions with CYP2D6.10, such as through increased steric hindrance. MAMP and MDMA, both being amphetamines, are similar structurally. It is not clear why the K_m of *p*-methoxyamphetamine, which is also related chemically to MAMP and MDMA, was not as significantly affected in CYP2D6.10. The presence of the methoxy group on PMA may act to increase its affinity for CYP2D6.10. MPTP was also less affected in its affinity for CYP2D6.10 in comparison to other substrates. Both compounds bear no obvious structural similarities, making further conclusions difficult. Since only a few substrates were studied, it is difficult to extrapolate the results to chemical structure determinants. Continued screening of different substrates and inhibitors is necessary to better evaluate characteristics of compounds which display significantly different catalytic activity in CYP2D6.10 compared to CYP2D6.1.

In addition to substrate-selectivity, enantiomeric differences in metabolism were observed with CYP2D6.10. MAMP exists in (+) and (−) enantiomers, with the (+) enantiomer being a more potent CNS stimulant (Cho, 1990). In CYP2D6.10 (−)MAMP *p*-hydroxylation was significantly more attenuated compared to CYP2D6.1 than (+)MAMP *p*-hydroxylation. Enantiomeric differences in methamphetamine metabolism have been previously demonstrated with CYP2D6.1, in that (−)MAMP has been shown to have a lower in-vitro intrinsic clearance than the (+) enantiomer for both NDM and *p*-hydroxylation (Lin *et al.*, 1997). The present results suggest such differences may also occur with CYP2D6.10. *In vivo*, one may expect *CYP2D6*10/*10* individuals to have higher plasma levels of the less potent (−)MAMP, compared to (+)MAMP, after ingesting (±)MAMP.

A common characteristic of substrates of CYP2D6 is that they possess one or more basic nitrogen atoms, with the site of oxidation of most CYP2D6 substrates residing 5–7 Å from the basic nitrogen (Koymans *et al.*, 1992). This structural requirement makes *N*-demethylation at the basic nitrogen an unlikely chemical reaction catalysed by CYP2D6, although exceptions have been observed, including amitriptyline and MPTP (Coutts *et al.*, 1994; Grace *et al.*, 1994; Coleman *et al.*, 1996; Rowland *et al.*, 1996; Ghahramani *et al.*, 1997; Olesen & Linnet, 1997). An alternative model involving differential positioning of the active site and electron transfer from the free base, has been proposed to account for these exceptions (Grace *et al.*, 1994). It was surprising, however, that amitriptyline, MPTP and MAMP which are *N*-demethylated at sites less than 5–7 Å from the basic nitrogen, were all significantly catalysed by CYP2D6.1. In fact, in CYP2D6.1-expressing microsomes, *N*-demethylation of (+) and (−)MAMP was more significant than the predicted *p*-hydroxy-

lation pathway. MAMP *N*-demethylation has been previously observed in yeast-expressing CYP2D6, at a higher rate than the corresponding *p*-hydroxylation reaction (Lin *et al.*, 1997). Moreover, a correlation between MAMP *N*-demethylation and metoprolol hydroxylation has been observed in a panel of human livers (Lin *et al.*, 1997). The high MAMP *N*-demethylation activity seen in expressed CYP2D6.1 is likely due to the high activity of the expression system. In addition, it may be that the slow hydroxylation of the aromatic ring of MAMP may allow the substrate to reposition in the catalytic site, making *N*-demethylation more likely. This so-called 'metabolic switching' has been previously proposed by others (Lin *et al.*, 1997).

In-vitro studies of CYP2D6 have demonstrated that the $Pro^{34}Ser$ amino acid substitution results in reduced enzyme expression in COS and yeast cells (Johansson *et al.*, 1994; Fukuda *et al.*, 2000), suggesting that, *in vivo*, CYP2D6.10 may be present in lower amounts that CYP2D6.1. These studies, combined with the reduced affinity and efficiency observed here with CYP2D6.10, indicate that individuals with *CYP2D6*10* would display decreased metabolism of these compounds *in vivo*. The substantial in-vitro differences between CYP2D6.1 and CYP2D6.10 suggest that reduced metabolism would be observed in both homozygotes and heterozygotes of *CYP2D6*10*. Consequently, increased concentrations of parent drug would result, which may translate into an increased risk of side-effects. These effects would be more likely if CYP2D6 plays a large role in the clearance of the substrate; and if the substrate has a narrow therapeutic index. CYP2D6 has been demonstrated to be the major enzyme involved in the metabolism of DEX, PMA and MDMA, and the *p*-hydroxylation of AMI (Kitchen *et al.*, 1979; Tucker *et al.*, 1994; Wu *et al.*, 1997; Nordin & Bertilsson, 1998; von Moltke *et al.*, 1998; Kreth *et al.*, 2000). Therefore, it is likely that those with *CYP2D6*10* will have significantly elevated plasma levels of these compounds. Indeed, Tateishi and coworkers demonstrated an approximate 50-fold decrease in DEX log MRs in *CYP2D6*10/*10* individuals relative to *CYP2D6*1/*1* individuals (Tateishi *et al.*, 1999). Given the likely importance of PMA O-demethylation *in vivo* (Kitchen *et al.*, 1979), the observed 35-fold reduced in-vitro PMA metabolism may result in a substantial decrease in PMA metabolism in *CYP2D6*10/*10* individuals. Since PMA is more centrally active than its metabolite, *p*-hydroxyamphetamine (Burde & Thompson, 1991), decreased metabolism of PMA by CYP2D6.10 indicates that *CYP2D6*10/*10* individuals may experience greater central stimulant effects at equal doses as those used by *CYP2D6*1/*1* individuals. Moreover, increased plasma PMA levels may result in enhanced systemic toxicity, because it produces marked and sustained elevations in blood pressure and hyperthermia in some users (Sellers *et al.*, 1979).

MDMA displayed a significant attenuation in intrinsic clearance in CYP2D6.10 (Table 2), to less than 1/120 the activity of CYP2D6.1. Therefore, one would expect significantly elevated plasma concentrations of MDMA in those with *CYP2D6*10*, relative to *CYP2D6*1/*1* individuals. Several deaths have been attributed to MDMA use, which appear to occur even in those taking average doses (Henry *et al.*, 1992). Reduced metabolism of MDMA, such as through CYP2D6.10, may result in toxic levels of MDMA, leading to cardiovascular and hyperthermic complications. In-vivo studies with MDMA in those of varied *CYP2D6* genotype are required to understand the effect of polymorphism on the pharmacokinetics and pharmacodynamics of MDMA.

Similar to MDMA (+) and (−)MAMP *N*-demethylation were significantly decreased in CYP2D6.10-expressing microsomes, as was *p*-hydroxylation, although to a lesser extent. The low intrinsic clearance observed presently in CYP2D6.1-expressing microsomes (Table 2) suggests that CYP2D6 may not be important in the overall clearance of MAMP. Data from pooled human liver microsomes indicated a similar low intrinsic clearance (data not shown), again suggesting a lack of importance of CYP450-catalysed reactions in overall MAMP clearance. The role of CYP2D6 polymorphism in MAMP metabolism and drug effect therefore remains to be established.

Given the reported minor role of *p*-hydroxylation in the clearance of AMI (Nordin & Bertilsson, 1998), and the inconclusive evidence of the role of CYP2D6 in its *N*-demethylation, the effects of CYP2D6 polymorphism on the pharmacokinetics of AMI are less clear. Similarly, the role of competing pathways in MPTP metabolism (Chiba *et al.*, 1988) may decrease the importance of the CYP2D6-catalysed *N*-demethylation reaction, though it has been shown that the rat model for the CYP2D6 poor metabolzers phenotype displayed a more pronounced and maintained deficit in motor activity after treatment with MPTP than the extensive metabolizer rat model (Jimenez-Jimenez *et al.*, 1991). Further characterization of the enzyme(s) involved in these reactions is necessary.

The SSRIs and amphetamines studied all displayed significantly different inhibition of CYP2D6.10, as compared to CYP2D6.1. Thus, one may expect that *in vivo*, *CYP2D6*10/*10* individuals would display decreased susceptibility to inhibition by these drugs. In contrast to the SSRIs and amphetamine analogues, budipine and sparteine inhibition constants

were minimally affected with CYP2D6.10 *in vitro*. These results suggest that these compounds are not especially constrained from interacting with the CYP2D6.10 active site. Since similar K_is were determined in CYP2D6.10 and CYP2D6.1 for these compounds, one may expect to observe similar inhibition profiles with these compounds *in vivo* in *CYP2D6*10/*10* and *CYP2D6*1/*1* individuals. One must bear in mind, however, that the degree of in-vivo inhibition also depends on factors other than K_i, including inhibitor concentration at the enzyme site and enzyme saturability. In addition, the significantly different effects observed with sparteine and debrisoquine in CYP2D6.10 suggest that the two may interact differentially as substrates with this enzyme, though further studies are necessary.

In conclusion, the present study indicates that CYP2D6.10 is an enzyme of both reduced affinity and efficiency compared to CYP2D6.1. Moreover, CYP2D6.10 displays substrate-, chemical pathway- and enantiomeric- selectivity with the compounds studied, indicating that extrapolating to metabolism of other substrates in individuals with this allele, relative to CYP2D6.1, may not be possible. Finally, these profound and variable reductions in CYP2D6.10-catalysed metabolism suggest that CYP2D6.10 should be taken into account during the drug development process.

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