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Reduced (±)-3,4-methylenedioxymethamphetamine (''Ecstasy'') metabolism with cytochrome P450 2D6 inhibitors and pharmacogenetic variants *in vitro*

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Abstract

''Ecstasy'' [(±)-3,4-methylenedioxymethamphetamine or MDMA] is a CNS stimulant, whose use is increasing despite evidence of long-term neurotoxicity. *In vitro*, the majority of MDMA is demethylenated to (±)-3,4-dihydroxymethamphetamine (DHMA) by the polymorphic cytochrome P450 2D6 (CYP2D6). We investigated the demethylenation of MDMA and dextromethorphan (DEX), as a comparison drug, in reconstituted microsomes expressing the variant *CYP2D6* alleles **2*, **10*, and **17*, all of which have been linked to decreased enzyme activity. With MDMA, intrinsic clearances (V_{max}/K_m) in CYP2D6.2, CYP2D6.17, and CYP2D6.10 were reduced 15-, 13-, and 135-fold, respectively, compared with wild-type CYP2D6.1. With DEX, intrinsic clearances were reduced by 37-, 51-, and 164-fold, respectively. It was evident that CYP2D6.17 displayed substrate-specific changes in drug affinity (K_m). Compounds potentially used with MDMA [fluoxetine, paroxetine, (−)-cocaine] demonstrated significant inhibition of MDMA metabolism in both human liver and CYP2D6.1-expressing microsomes. These data demonstrate that individuals possessing the *CYP2D6*2*, **17*, and, particularly, **10* alleles may show significantly reduced MDMA metabolism. These individuals, and those taking CYP2D6 inhibitors, may demonstrate altered acute and/or long-term MDMA-related toxicity.

Keywords: (±)-3,4-Methylenedioxymethamphetamine; Cytochrome P450 2D6; Drug metabolism; Pharmacogenetics; Drug interactions; Pharmacokinetics

1. Introduction

MDMA (''Ecstasy'', ''Adam'') is a synthetic amphetamine derivative that is being used increasingly for its stimulant and enactogenic properties, particularly in the rave culture [1]. The popularity of MDMA has led to an increased awareness of its potential toxicity. Acute 5-HT and 5-HIAA depletion has been observed in rat and non-human primate models [2–4]. In humans, acute MDMA toxicity manifests as (a) increased heart rate and blood pressure, and (b) hyperthermia, potentially leading to rhabdomyolysis, kidney and liver failure, and death [5]. In addition to acute overdose, MDMA is linked to chronic changes in serotonergic function [4]. In rats and non-human primates, decreases in tryptophan hydroxylase activity and the number of [^{3}H]paroxetine-binding sites, as well as 5-HT neurodegeneration, have been observed for up to 18 months after MDMA administration [4]. In humans, a global decrease in 5-HT transporter density was observed in heavy MDMA users, leading to potential behavioral and psychological abnormalities [4,6–8]. The

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Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; DHMA, 3,4-dihydroxymethamphetamine; MAMP, methamphetamine; PMA, *p*-methoxyamphetamine; MMDA-2, 2-methoxy-4,5-methylenedioxyamphetamine; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; SOD superoxide dismutase; DEX, dextromethorphan; DOR, dextrorphan; HLM, human liver microsomes; and DLPTC, L-α-phosphatidylcholine, dilauroyl.

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mechanism(s) of MDMA-induced neurotoxicity is not yet established. Current hypotheses relate to: oxidative stress, toxic metabolites of MDMA and/or neurotransmitters, and glutamate mediation (for review, see Ref. [9]).

The polymorphic enzyme cytochrome P450 2D6 (CYP2D6) is involved in the metabolism of a broad array of drugs, including antidepressants and various amphetamine analogues [10–13]. In addition to null alleles causing CYP2D6 poor metabolizer status, several *CYP2D6* alleles causing reduced enzymatic activity exist. The variant enzymes CYP2D6.10 (P34S and S486T amino acid mutations), CYP2D6.17 (T107I, R296C, S486T), and CYP2D6.2 (R296C, S486T) have been linked to decreased enzymatic activity *in vitro* and *in vivo* [14–20]. These alleles differ significantly in their interethnic prevalence, with *CYP2D6*10* allele prevalence being particularly high in Asians (50%), *CYP2D6*17* in Black Africans (34%), and *CYP2D6*2* in Ethiopians (10-29%) and Caucasians (25-35%) [15,20–23].

MDMA is both demethylenated to DHMA, and demethylated to MDA [24,25]. CYP2D6 has been shown to be the major demethylenase at low MDMA concentrations *in vitro* [26,27]. *In vivo*, it has been observed that 4-hydroxy-3-methoxymethamphetamine, the *O*-methylated metabolite of DHMA, is the major product in human plasma after moderate MDMA doses, with MDA being relatively minor [28], suggesting that demethylenation may be the major *in vivo* metabolic pathway of MDMA.

Given the importance of drug metabolism in determining the magnitude of the effect(s) of a drug, reduced MDMA metabolism may impact the likelihood of adverse consequences in particular drug-using individuals. Our main objectives, therefore, were (a) to evaluate MDMA metabolism in human liver and reconstituted microsomes expressing the reduced-activity *CYP2D6* alleles **17*, **10*, and **2*; and (b) to determine CYP2D6 metabolic interactions with potentially co-administered drugs, including (−)-cocaine and various selective serotonin reuptake inhibitors.

2. Materials and methods

2.1. Chemicals

DLPTC and quinine were purchased from Sigma Chemical Co. (−)-Cocaine, MMDA-2, (+)-MAMP, PMA, and (+)-, (−)-, and (±)-MDMA were obtained from the National Institute on Drug Dependence. Dextromethorphan hydrobromide and dextrorphan tartrate were provided by Hoffmann-La Roche Inc. Citalopram hydrobromide was purchased from H. Lundbeck A/S. Butorphanol tartrate, tryptamine, β-NADPH sodium salt, and SOD were purchased from Sigma. (±)-Fluoxetine and (±)-norfluoxetine maleate were obtained from the Eli Lilly Co. Sertraline hydrochloride was obtained from Parke-Davis. (±)-DHMA was provided by Dr. Arthur Cho. HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific. Perchloric acid was obtained from Caledon Laboratory Chemicals. Potassium phosphate monobasic and heptane sulfonic acid were ACS-grade.

2.2. cDNA-expressing microsomes

Microsomes (CYPs 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) from human lymphoblast cells as well as CYP2D6.1-expressing microsomes from BTI-TH-5B1-4 insect cells were purchased from the GENTEST Co. Purified enzymes (CYP2D6.1, CYP2D6.2, CYP2D6.17, and CYP2D6.10) were expressed in *Trichoplusia ni* (*T. ni*) cells via a baculovirus vector [29], and their activity was reconstituted as described below.

2.3. Human Liver Microsomes (HLM)

The K-series liver (K25) (genotyped as *CYP2D6*1/*1*) used in this study was described previously [30,31]. Pooled microsomes were purchased from GENTEST Co. Details on the backgrounds of the liver donors are provided in the GENTEST catalogue and inserts obtained with the products. HL-series liver microsomes were provided by the Department of Medicinal Chemistry, University of Washington.

2.4. HPLC

A Hewlett-Packard 1100 isocratic pump, with a 1050 autosampler, 1050 UV detector and an HP339611 integrator were used for drug analyses.

2.5. Substrate assays

Incubations were carried out in 0.1 M KH_2PO_4 buffer (pH 7.4) at 37° under continuous shaking. Substrate, buffer, and 1 mM NADPH were mixed in a final volume of 200 μL, and the reactions were initiated by the addition of enzyme. For (±)-MDMA studies, 30 units of SOD was added to minimize the conversion of DHMA to its *o*-quinone.

2.6. MDMA screening in cDNA-expressing microsomes

cDNA-expressing CYPs from human lymphoblast cells were incubated with 10 μM MDMA, buffer, SOD, and NADPH for 25 min. These CYPs had varying amounts of activity (with respect to the metabolism of their CYP-specific probe drug) relative to HLM, which was measured and provided by GENTEST. Activities of the cDNA-expressing microsomes were normalized by diluting protein concentration to account for these differences. A final protein concentration of 0.48 mg/mL and an incubation time of 25 min were used for kinetic studies with cDNA-expressing CYP2D6.

2.7. CYP2D6*1, -*2, -*17, and -*10-expressing microsomes

As NADPH reductase was not co-expressed with the CYP alleles, catalytic activity was reconstituted using lipid (DLPTC) and NADPH reductase. The optimal ratio of P450:reductase:lipid and the incubation procedure, using DEX as a substrate, were determined previously. Final DEX concentrations of 1–500 μM and final MDMA concentrations of 1.95–500 μM were used. The general reconstitution and incubation procedure was as follows: CYP enzyme and reductase were co-incubated at room temperature for 15 min. DLPTC was then added to the mixture and incubated at room temperature for another 10 min. Next, substrate, buffer, and SOD (for MDMA only) were added and incubated for 5 min. NADPH was added to initiate the reactions, which proceeded at 37° for the incubation times listed in Table 1 (the concentrations of each reagent added are also given in this table). Preliminary studies were performed, using DEX, to determine incubation conditions under which DOR production was linear with respect to time and CYP concentration. Butorphanol and tyramine were used as internal standards for DEX and MDMA studies, respectively.

2.8. HLM studies

DHMA production was linear for up to 0.8 mg/mL of protein in HLM and for up to 30 min of incubation time. Therefore, 0.4 mg/mL and 25 min were chosen as incubation conditions for K-series and pooled HLM. Since HL-series livers were, in general, more active, a shortened incubation time of 15 min was used for these studies. Final MDMA concentrations of 0.613 to 500 μM were used. DOR production was linear for up to 0.8 mg/mL and 45 min of incubation. A protein concentration of 0.2 mg/mL was used. Incubations proceeded for 20 and 10 min, for K-25 and HL-series livers, respectively, with 0.5 to 500 μM DEX.

2.9. Inhibition assays

MDMA was incubated at final concentrations of K_m, $2K_m$, and $4K_m$ for K-25 HLM and CYP2D6.1. These concentrations were chosen since the peak size was too small to inhibit at the $0.5K_m$ of MDMA. Therefore, the final MDMA concentrations used for HLM were 2, 4, and 8 μM. For CYP2D6.1, final MDMA concentrations were 1.5, 3, and 6 μM. DHMA production was monitored in the presence and absence of four different concentrations of each chemical inhibitor. All inhibition screening studies involved inhibitor concentrations of 1 and 10 μM (performed in duplicate), which were incubated with $4K_m$ (6 μM) of MDMA. All inhibitors were dissolved in Millipore-filtered water except for (−)-cocaine, which was dissolved in methanol. The methanol was then evaporated under nitrogen to dryness and redissolved in the appropriate volume of buffer. The reactions were initiated by the addition of 0.4 mg/mL of K-25 liver microsomes or 1.5 pmol of CYP2D6.1 and proceeded for 25 and 10 min, respectively.

2.10. Extraction

2.10.1. DEX

The extraction procedure was essentially that of Chen *et al.* [32]. 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 (3000 *g*, 4°) for 10 min each, and back-extracted with 100 μL of 0.01N 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)].

2.10.2. MDMA

The reactions were stopped by the addition of 15 μL of 70% perchloric acid. Samples were vortexed and centrifuged (3000 *g*, 4°) for 20 min to pellet the protein. Then 70 μL of the top layer was 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.

2.11. Data analyses

Kinetic analyses involving one-site kinetics were performed using Enzfitter software. Kinetic analyses involving two-site kinetics were performed using Graphpad software version 2.01. Dixon and Cornish-Bowden plots were generated using Microsoft Excel.

Table 1
Experimental parameters used for incubations in reconstituted microsomes from *T. ni* cells expressing various *CYP2D6* alleles

Substrate	*CYP2D6* allele	CYP amount (pmol)	NADPH reductase (μM)	DLPTC (μg)	Final incubation time (min)
DEX	*1	3.4	0.35	0.03	4
	*2, *10, and *17	13.0	1.32	0.10	25
MDMA	*1	3.4	0.35	0.03	25
	*2, *10, and *17	13.0	1.32	0.10	40

Table 2
Summary of kinetic parameters of DEX and MDMA metabolism in human liver microsomes

Human liver microsomes	Substrate	Site	K_m (μM)	V_{max} (nmol/mg/min)	Intrinsic clearance (V_{max}/K_m) (mL/mg/min)
K-25	DEX	1	3.6	0.1	0.03
	MDMA	1st	1.5	0.007	0.005
		2nd	607.6	0.04	0.0007
Pooled	MDMA	1st	2.0	0.005	0.003
		2nd	398.5	0.06	0.0001

Data shown are the result of one determination.

2.12. Statistical analyses

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 four substrate/inhibitor–enzyme combinations. The log ratios of K_m in *10, *17, or *2 to K_m in *1 were sorted and compared using the Duncan New Multiple Range Test ($P < 0.05$), allowing determination of significant pairwise differences.

3. Results

3.1. MDMA demethylenation in HLM

As shown in Table 2, two-site kinetics were determined in K-25 and pooled HLM, with similar K_m and V_{max} values. To investigate the degree of MDMA metabolism relative to a typical CYP2D6 probe, DEX was used as a comparison substrate in K-25 HLM. In K-25 HLM, DEX *in vitro* intrinsic clearance was approximately 6.5-fold higher than MDMA intrinsic clearance, indicating MDMA demethylenation is not catalyzed by CYP2D6 as efficiently as is DEX in HLM.

3.2. MDMA demethylenation by cDNA-expressing CYPs

To investigate the relative involvement of different CYPs in the high-affinity site of (±)-MDMA demethylenation, screening experiments were performed with cDNA-expressing microsomes from human lymphoblast cells. At 10 μM MDMA, only CYP2D6 produced DHMA (data not shown), indicating that CYP2D6 is the sole MDMA demethylenase at low (±)-MDMA concentrations. K_m and V_{max} values in cDNA-expressing CYP2D6 were 4.1 ± 0.59 μM and 0.25 ± 0.12 nmol/mg/min, respectively, demonstrating a similar affinity for (±)-MDMA as in HLM.

3.3. MDMA metabolism in microsomes expressing various CYP2D6 alleles

DEX and MDMA metabolism was evaluated in reconstituted microsomes from *T. ni* cells expressing the *CYP2D6*1*, -*2, -*10, and -*17 alleles. All MDMA kinetic analyses were performed in duplicate except for CYP2D6.10, and all DEX analyses were performed singly. Table 3 summarizes the K_m, V_{max}, and *in vitro* intrinsic clearance values obtained. Incubations conducted in the absence of lipid and reductase failed to produce either DOR or DHMA (data not shown). With both DEX and MDMA as substrates, CYP2D6.1 displayed monophasic kinetics. For MDMA, CYP2D6.2, CYP2D6.10, and CYP2D6.17 all displayed biphasic kinetics. For DEX, CYP2D6.2 and CYP2D6.17 displayed biphasic kinetics (Fig. 1), while CYP2D6.10 displayed monophasic kinetics. Since the *in vitro* intrinsic clearances for the low-affinity sites were very low (below 1.4×10^{-6}), only the high-affinity site kinetics are displayed and compared in Table 3. As shown, the metabolism of both DEX and MDMA was reduced with all three variant alleles in comparison to CYP2D6.1, with CYP2D6.10 displaying the lowest

Table 3
Summary of kinetic parameters of DEX and MDMA with CYP2D6.1, CYP2D6.2, CYP2D6.17 and CYP2D6.10 reconstituted microsomes

Substrate	*CYP2D6* allele	K_m (μM)	V_{max} (nmol/pmol/min) ($\times 10^{-5}$)	Intrinsic clearance (mL/pmol 2D6/min) ($\times 10^{-5}$)	Ratio of intrinsic clearance in .1 to .2, .17, or .10[a]
DEX	*1	4.0	328.0	81.8	
	*2	13.6	30.3	2.2	37.2
	*17	60.8	96.7	1.6	51.1
	*10	27.8	12.6	0.5	163.6
MDMA	*1	6.2 ± 2.6[b]	167.5 ± 9.2[b]	30.3 ± 144[b]	
	*2	10.3 ± 3.5[b]	19.0 ± 9.0[b]	1.8 ± 0.3[b]	15.0
	*17	3.5 ± 1.5[b]	7.5 ± 1.8[b]	2.6 ± 1.6[b]	12.9
	*10	18.7	4.4	0.2	135.0

[a] Ratio equals (V_{max}/K_m in CYP2D6.1)/(V_{max}/K_m in CYP2D6.17, CYP2D6.10, or CYP2D6.2).
[b] Means (± range) for two independent determinations.

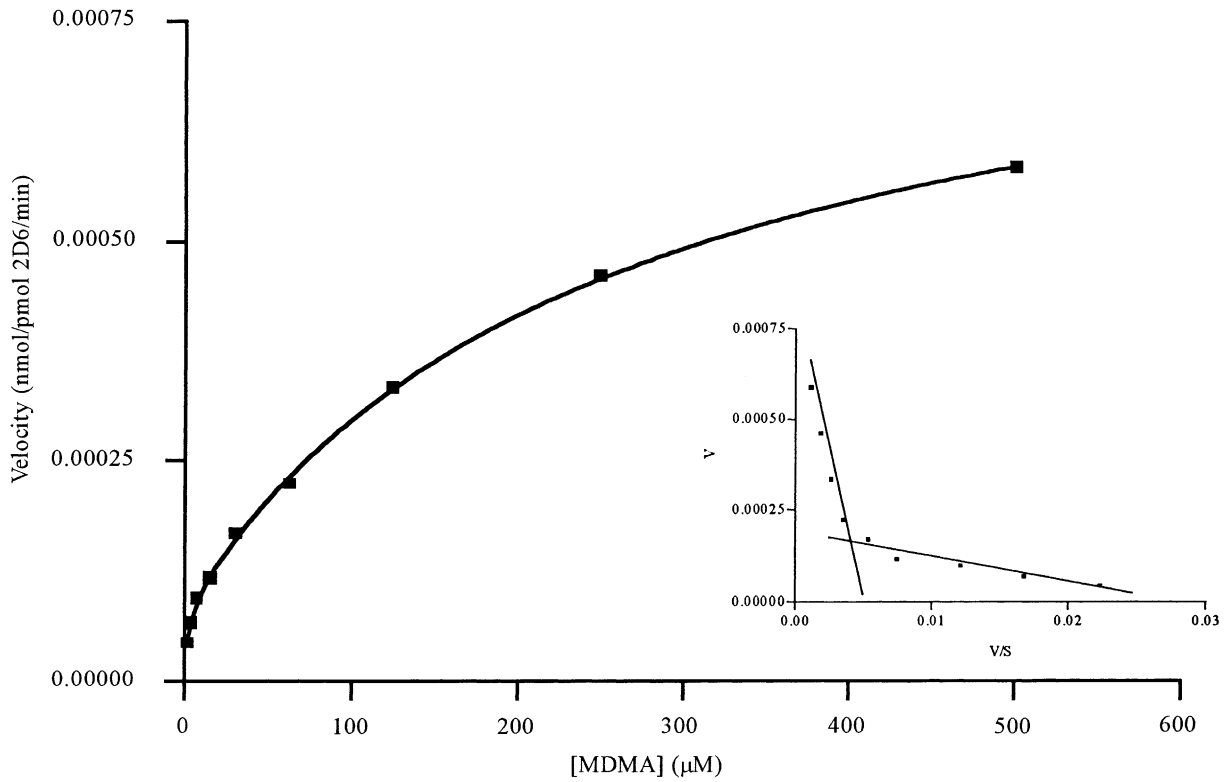

Fig. 1. Michaelis–Menten curve of MDMA demethylenation in reconstituted microsomes from *T. ni* cells expressing CYP2D6.17. Inset: Eadie-Hofstee plot demonstrating biphasic kinetics. Data shown represent one of two independent determinations.

intrinsic clearances for both DEX and MDMA. The order of activity for DEX was CYP2D6.1 > CYP2D6.2 > CYP2D6.17 > CYP2D6.10. The order of activity for MDMA was CYP2D6.1 > CYP2D6.17 > CYP2D6.2 > CYP2D6.10.

3.4. DEX and MDMA metabolism in HLM expressing *CYP2D6*2* and *CYP2D6*10*

DEX and MDMA metabolism was also evaluated in HLM expressing the **2* and **10* alleles heterozygously. *CYP2D6*2* itself is linked to decreased enzyme activity, but in multiple copies it is linked to ultrarapid enzyme activity. However, the number of *CYP2D6*2* alleles in these livers was not determined. Only the high-affinity site kinetics are shown in Table 4. With MDMA, K_m, V_{max}, and intrinsic clearance values were similar across the different livers and genotypes, with the exception of the *1/*2 livers (HL-143, HL-156), which showed approximately 20–40% of the average activity of the wild-type livers (HL-160, HL-139). With DEX, similar kinetic constants were again seen across the various livers, with intrinsic clearances akin to those seen with MDMA. As with MDMA, *1/*2 livers displayed the lowest intrinsic clearance (Table 4). In contrast to the previous HLM results (Table 2), no significant differences were seen between DEX and MDMA metabolism in *1/*1 livers.

3.5. Interactions of MDMA with CYP2D6 inhibitors

Ten known CYP2D6 inhibitors (at 1 and 10 μM) were used to inhibit MDMA demethylenation in CYP2D6.1-

Table 4
Kinetic parameters of DEX and MDMA in human liver microsomes expressing *CYP2D6*1*, **2* and **10* alleles

CYP2D6 genotype	MDMA[a]			DEX		
	K_m (μM)	V_{max} (nmol/mg/min)	Intrinsic clearance (V_{max}/K_m) (mL/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)	Intrinsic clearance (V_{max}/K_m) (mL/mg/min)
*1/*1 (HL-160[b], HL-139)	0.9 ± 0.1	0.01 ± 0.003	0.02 ± 0.006	2.2 ± 0.7	0.05 ± 0.02	0.03 ± 0.01
*1/*10 (HL-135[b], HL-132)	1.0 ± 0.6	0.01 ± 0.005	0.01 ± 0.003	2.5 ± 1.2	0.04 ± 0.01	0.02 ± 0.005
*2/*10 (HL-142[b], HL-133)	0.7 ± 0.07	0.01 ± 0.003	0.02 ± 0.007	2.8 ± 0.2	0.04 ± 0.02	0.02 ± 0.008
*1/*2 (HL-143[b], HL-156[b])	1.6 ± 0.6	0.007 ± 0.001	0.004 ± 0.003	1.9 ± 0.1	0.02 ± 0.006	0.01 ± 0.004

Means (± range) are reported for single determinations from two different livers, listed in parentheses after each genotype group.
[a] Two-site kinetics observed for all livers with MDMA.
[b] Two-site kinetics indicated for DEX metabolism.

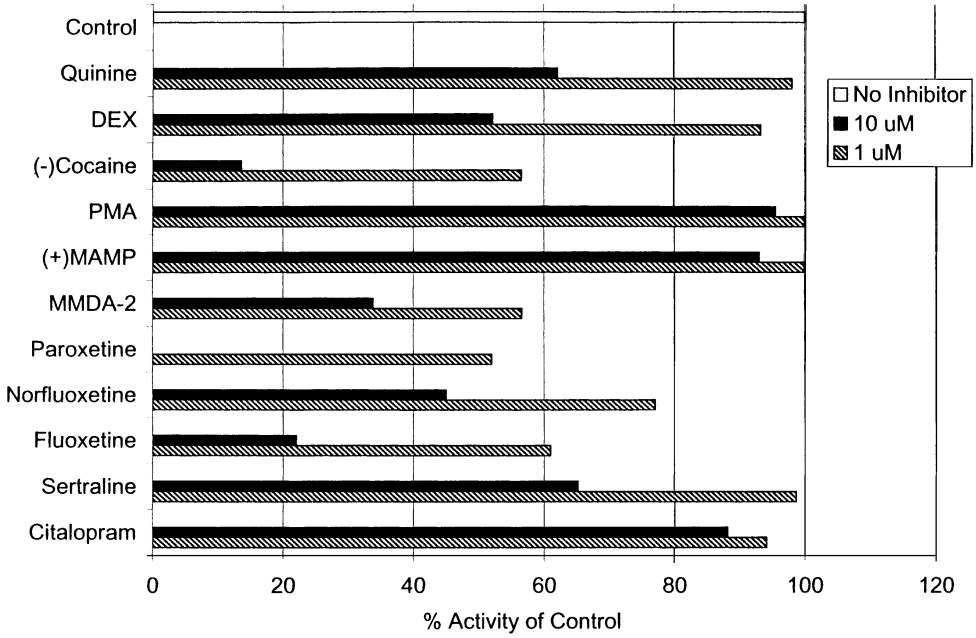

Fig. 2. Inhibition screening of MDMA demethylenation in CYP2D6.1-expressing microsomes from insect cells. Percent activity of control refers to the percentage of remaining MDMA demethylenation in the presence of inhibitor relative to the percentage of demethylenation in the absence of inhibitor (control). Each bar represents the mean of two replicate determinations.

expressing microsomes obtained from GENTEST Co. The inhibitors tested were: the selective serotonin reuptake inhibitors (SSRIs) citalopram, sertraline, fluoxetine, norfluoxetine, and paroxetine; the amphetamines MMDA-2, (+)-MAMP, and PMA; the antitussive DEX; and the CNS stimulant (−)-cocaine. Quinine was used as a negative control of CYP2D6 inhibition. Figure 2 shows the effects of these compounds on MDMA demethylenation. Among the SSRIs, paroxetine, fluoxetine, and norfluoxetine showed over 50% inhibition, relative to control, at 10 μM, with sertraline and citalopram having weaker effects. With the amphetamines, only MMDA-2 showed demonstrable MDMA inhibition (almost 70%) at a 10 μM concentration. In addition, (−)-cocaine inhibited MDMA demethylenation by approximately 85% at a concentration of 10 μM.

K_i values were determined for the compounds showing the strongest inhibition upon screening in K-25 HLM and cDNA-expressing microsomes. Paroxetine K_i values were determined in triplicate in expressed-CYP2D6.1. All other K_i values were determined singly. Table 5 summarizes the K_i values obtained. Except for norfluoxetine, all compounds potently inhibited MDMA demethylenation in HLM, with the ascending order of inhibition constants (K_i values) being paroxetine < (−)-cocaine < fluoxetine < norfluoxetine. In cDNA-expressing microsomes, the ascending K_i order was (−)-cocaine < paroxetine < fluoxetine. Norfluoxetine was not evaluated because of the low inhibition constant determined in HLM. All compounds displayed competitive inhibition, as determined by Cornish-Bowden plots (data not shown).

Table 5
Summary of inhibition of MDMA metabolism in K-25 human liver and CYP2D6.1-expressing microsomes from insect cells

Inhibitor	K_i human liver microsomes (μM)	K_i CYP2D6.1 (μM)
Paroxetine	0.5	0.4 ± 0.2[a]
Fluoxetine	2.8	0.8
Norfluoxetine	10.0	ND[b]
(−)-Cocaine	2.2	0.2

[a] Average (± SD) of three independent determinations.
[b] ND: not determined.

4. Discussion

The present results demonstrate that HLM display a high specificity for MDMA, as evidenced by the low K_m values obtained, as has been demonstrated previously [26,27]. However, based on the ∼6-fold reduction in intrinsic clearance obtained in pooled HLM, MDMA may not be metabolized as efficiently as DEX in HLM. Screening experiments in cDNA-expressing CYPs from human lymphoblast cells indicated that CYP2D6 is the sole CYP involved in MDMA demethylenation at low MDMA concentrations typically present in plasma [33], in line with previous studies [26,27,34].

The similar kinetic profiles seen with the HL-series human livers (Table 4) across genotypes suggests that *CYP2D6*2* or *CYP2D6*10* alleles present heterozygously do not alter MDMA or DEX metabolism significantly, relative to *CYP2D6*1/*1* livers. Lower intrinsic clearance values were observed with the *CYP2D6*1/*2* livers, than

with livers expressing both *CYP2D6*2* and *CYP2D6*10*. It may be that *CYP2D6*2* is present in multiple copies in the HL-142 and HL-133 livers, or that the *CYP2D6*1/*2* livers are simply of lower intrinsic activity.

cDNA-expressing CYP2D6.2, CYP2D6.17, and CYP2D6.10 all displayed decreased V_{max} values and *in vitro* intrinsic clearances for DEX and MDMA, as compared with CYP2D6.1. *In vivo*, CYP2D6.2/.2 individuals have been shown to have higher activity than CYP2D6.17/.17 individuals, using DEX as the probe drug [19]. As shown in Table 3, CYP2D6.2 showed the lowest attenuation in intrinsic clearance, as compared with CYP2D6.1, using DEX as the substrate. However, CYP2D6.2 displayed a slightly lower intrinsic clearance than CYP2D6.17 with MDMA. The differences in intrinsic clearance between DEX and MDMA with CYP2D6.2 were statistically significant ($P < 0.05$), illustrating substrate-specificity in the degree of metabolism. In addition, the approximately 3.5- and 1.7-fold increases in K_m values (relative to CYP2D6.1) with DEX and MDMA, respectively, demonstrates altered substrate affinity with CYP2D6.2. These data suggest that the amino acid mutations present in this enzyme alter substrate binding, akin to CYP2D6.17 and CYP2D6.10. CYP2D6.2 shares the R296C mutation with CYP2D6.17 and the S486T mutation with both CYP2D6.10 and CYP2D6.17, both of which have demonstrated altered drug affinities [16,17]. Both decreased and normal metabolism have been observed in individuals with CYP2D6.2 when given different CYP2D6 substrates [19,35].

CYP2D6.17 displayed the most varied intrinsic clearance values between the two substrates. With DEX, intrinsic clearance was ~50-fold lower in CYP2D6.17 than in CYP2D6.1, versus an ~13-fold attenuation with MDMA. These changes were more reflective of a pronounced increase in K_m for DEX in CYP2D6.17, relative to CYP2D6.1, compared with a reduction in K_m for MDMA. Such K_m differences have been noted previously *in vitro* [17] and are suggestive of differential interactions of DEX and MDMA in the substrate pocket of CYP2D6.17. The demonstrated substrate-selectivity in intrinsic clearance and drug affinity observed with CYP2D6.17 suggest that this enzyme may interact differentially with different CYP2D6 substrates *in vivo*. Indeed, *in vivo*, CYP2D6.17 has been linked to differential metabolism of multiple CYP2D6 probe drugs [18]. With both CYP2D6.17 and CYP2D6.2, additional substrates, as well as inhibitors, should be investigated to better address these issues of selectivity and altered metabolism.

CYP2D6.10 displayed the least activity with both DEX and MDMA, at ~163.6- and ~135-fold the intrinsic clearance of CYP2D6.1, respectively. This decrease reflected more profound decreases in $V_{max,}$ than in affinity. The mutated Pro34 in CYP2D6.10 is in an important proline-rich region linked to protein conformation and stability, a possible reason for the low V_{max} values observed.

Since microsomes that were not reconstituted with reductase and lipid failed to produce either DOR or DHMA, as did incubations without either substrate, it is unlikely that the low-affinity site observed with CYP2D6.2, CYP2D6.10, and CYP2D6.17 arose from non-enzymatic metabolite formation. It is possible that two distinct substrate-binding sites are present in the active site of these variant CYP2D6 enzymes in the reconstituted system used, each possessing different affinities for the substrates, resulting in biphasic kinetics. These two sites may interact in a cooperative manner, or independently. Korzekwa and coworkers [36] demonstrated a similar biphasic saturation profile to that observed here, using naproxen with CYP2C9-expressing microsomes. Evidence for two distinct substrate-binding sites has also been obtained with CYP3A4-expressing microsomes with selected substrates [37]. The possibility of multiple binding sites in CYP2D6 variants in the cell system used requires further investigation.

Because of the more profound attenuation in intrinsic clearance seen with CYP2D6.10, the decreases in MDMA demethylenation *in vivo* with CYP2D6.2 and CYP2D6.17 may not be as large as that with CYP2D6.10. However, *in vivo* metabolism will also depend on the expression level of the enzyme, which may vary with different CYP2D6 variants. Nonetheless, the dominance of CYP2D6 in the demethylenation pathway demonstrated *in vitro*, combined with evidence that this pathway may contribute to a large proportion of MDMA clearance *in vivo* [28], implies that the approximate 15-fold decrease in intrinsic clearance seen with CYP2D6.2 and CYP2D6.17 *in vitro* may translate into a significant attenuation in MDMA metabolism *in vivo*. Increased levels of MDMA may, in turn, lead to an increased risk for acute MDMA toxicity, as MDMA plasma levels have been shown to correlate with certain clinical effects, such as blood pressure and heart rate [33].

The SSRIs fluoxetine and paroxetine may be used by individuals either before or after the ingestion of MDMA. Presumably, consuming an SSRI after MDMA may prevent long-term serotonergic damage by preventing MDMA uptake into the 5-HT (serotonergic) neuron where it may cause damage. (−)-Cocaine, as part of (±)-cocaine, is also a stimulant drug that may be co-administered with MDMA by multi-drug users. These drugs were shown to be potent inhibitors of MDMA demethylenation in both human liver and CYP2D6.1-expressing microsomes, with K_i values in the micromolar and submicromolar range. Therefore, the use of these drugs with MDMA would be expected to increase plasma and CNS MDMA levels, which may contribute to altered toxicity profiles. Since paroxetine has a considerably shorter half-life than fluoxetine, and is a more potent CYP2D6 inhibitor, one may expect more significant but less sustained inhibition of MDMA metabolism when using paroxetine, compared with fluoxetine. It appears that the amphetamines PMA and (+)-MAMP, as well as the SSRIs sertraline and citalopram, do not inhibit MDMA demethylenation significantly.

In conclusion, the significantly reduced MDMA demethylenation demonstrated *in vitro* with CYP2D6.2, CYP2D6.17, and, in particular, CYP2D6.10 suggests that the pharmacokinetics, pharmacodynamics, and, potentially, the toxicity of this stimulant drug may vary according to *CYP2D6* genotype and ethnicity. Preliminary findings also indicate that drugs that may be co-administered with MDMA, particularly some SSRIs, may inhibit MDMA demethylenation significantly in the drug user. A better understanding of the role of demethylenation in the overall clearance of MDMA *in vivo* should help in predicting the clinical impact of these pharmacokinetic changes.

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