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Interactions of Amphetamine Analogs with Human Liver CYP2D6

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ABSTRACT. The interaction of fifteen amphetamine analogs with the genetically polymorphic enzyme CYP2D6 was examined. All fourteen phenylisopropylamines tested were competitive inhibitors of CYP2D6 in human liver microsomes. The presence of a methylenedioxy group in the 3,4-positions of both amphetamine (K_i = 26.5 μM) and methamphetamine (K_i = 25 μM) increased the affinity for CYP2D6 to 1.8 and 0.6 μM, respectively. Addition of a methoxy group to amphetamine in the 2-position also increased the affinity for CYP2D6 (K_i = 11.5 μM). The compound with the highest affinity for CYP2D6 was an amphetamine analog (MMDA-2) having both a methoxy group in the 2-position and a methylenedioxy group (K_i = 0.17 μM). Mescaline did not interact with CYP2D6. O-Demethylation of *p*-methoxyamphetamine (PMA) by CYP2D6 was characterized (K_m = 59.2 ± 22.4 μM, and V_{max} = 29.3 ± 16.6 nmol/mg/hr, N = 6 livers). This reaction was negligible in CYP2D6-deficient liver microsomes, was inhibited stereoselectively by the quinidine/quinine enantiomer pair, and was cosegregated with dextromethorphan O-demethylation (r = 0.975). The inhibitory effect of methylenedioxymethamphetamine (MDMA) was enhanced by preincubation with microsomes, suggesting that MDMA may produce a metabolite complex with CYP2D6. These findings suggest that phenylisopropylamines as a class interact with CYP2D6 as substrates and/or inhibitors. Their use may cause metabolic interactions with other drugs that are CYP2D6 substrates, and the potential for polymorphic oxidation via CYP2D6 may be a source of interindividual variation in their abuse liability and toxicity. BIOCHEM PHARMACOL **53**;11:1605–1612, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. CYP2D6; amphetamine; MDMA; human liver microsomes; methamphetamine; *in vitro* screening

Genetic polymorphism of the human CYP2D6¶ enzyme is a major source of pharmacokinetic variation for more than thirty clinically used drugs [1–4]. CYP2D6 activity is absent in about 7% of Caucasians owing to CYP2D6 gene mutations or deletion [5]. Such people are phenotyped as PMs, whereas the rest of the population (EMs) metabolize these drugs to various extents. In EMs, CYP2D6 is also an important site of drug interactions, either between competing CYP2D6 substrates, or between CYP2D6 substrates and drugs that are potent inhibitors of the enzyme but are not themselves significantly metabolized by this enzyme, e.g. quinidine [6, 7] or fluoxetine [8].

CYP2D6 substrates include tricyclic antidepressants, lipophilic β-adrenoceptor blockers, methoxymorphinans, and several amphetamine analogs, including amphetamine itself. Indeed, deficiency in the 4-hydroxylation of amphetamine [9, 10] and the O-demethylation of PMA were two early observations that led to the discovery of the CYP2D6 polymorphism. A PM subject was observed to excrete 4.4% of a 5 mg oral dose of PMA as 4-hydroxyamphetamine, compared to 50 and 65% excreted as this metabolite in two EMs [11]. PMA has amphetamine-like CNS stimulant effects and hallucinogenic properties [12, 13]. We speculate that phenotypic variation in its metabolism may have contributed to several deaths [14, 15] associated with PMA use. Recently, Tucker *et al.* [16] reported that MDMA ("Ecstasy") is demethylenated by CYP2D6 *in vitro*. Demethylenation of MDMA to the reactive catechol metabolite [17], and aromatic hydroxylation and demethylenation to a 6-hydroxydopamine analog [18] are two routes proposed to give rise to neurotoxic MDMA metabolites. Thus, the neurotoxic effects of MDMA may be associated with CYP2D6 activity. In the case of (+)-amphetamine, CYP2D6-mediated 4-hydroxylation is a minor pathway in humans. However, studies in animals suggest that pharmacologically active secondary metabolites are formed. 4-Hy-

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¶ *Abbreviations:* CYP2D6, cytochrome P450 2D6; EM, extensive metabolizer; PM, poor metabolizer; PMA, *p*-methoxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MMDA-2, 2-methoxy-4,5-methylenedioxyamphetamine; MDA, 3,4-methylenedioxyamphetamine; MMDA, 3-methoxy-4,5-methylenedioxyamphetamine; 3,4,5-TMA, 3,4,5-trimethoxyamphetamine; and 2,4,6-TMA, 2,4,6-trimethoxyamphetamine.

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droxyamphetamine is converted by dopamine-β-hydroxylase to *p*-hydroxynorephedrine, a "false-neurotransmitter" [19, 20], and it is also metabolized to α-methyldopamine and further to α-methylnoradrenaline, which may also function as a "false-neurotransmitter" [21]. 4-Hydroxyamphetamine itself has been used topically in ophthalmology for its sympathomimetic properties [22]. Unlike amphetamine, it has few, if any, central effects, presumably because its relative water solubility slows its passage through the blood-brain barrier [22]. However, CYP2D6 occurs in human brain [23, 24], and hydrophilic metabolites formed centrally may contribute to drug effects or toxicity. Amphetamine 4-hydroxylation [25] and MDMA demethylenation [26] have been demonstrated in rat brain microsomes.

It seems likely that phenylisopropylamines as a drug-class are substrates of CYP2D6. The present study examined the extent to which a series of such compounds interact with a recombinant form of CYP2D6 and CYP2D6 in human liver microsomes.

MATERIALS AND METHODS

Chemicals

Dextromethorphan hydrobromide, quinidine hydrochloride, quinine hydrochloride, D-glucose-6-phosphate monosodium salt, NADP sodium salt, and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St. Louis, MO). Dextrorphan tartrate and levallorphan tartrate (internal standard) were provided by Hoffmann–La Roche Inc. (Nutley, NJ). Fifteen amphetamine analogs were obtained from the National Institute on Drug Abuse, Research Technology Branch (Rockville, MD). Their chemical structures are listed in Table 1. All were in the form of HCl salts, except for 4-hydroxyamphetamine, which was the HBr salt. All other chemicals were purchased from Sigma.

Human Liver Samples and Preparation of Liver Microsomes

The characteristics of the fourteen human livers obtained from kidney transplant donors were described previously [27, 28]. Microsomes from these tissues were prepared by standard techniques [27]. Microsomal protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL) with the bovine serum albumin standard solution provided. CYP2D6 activity was detectable in twelve of the livers using sparteine as the substrate [28], while studies using immunochemical [29] and molecular [30, 31] techniques have shown two of the livers (K16 and K19) to be from PM donors.

CYP2D6 in a Yeast Expression System

Microsomes from yeast transformed with an expression plasmid containing full-length human CYP2D6 cDNA and microsomes from control yeast cells transfected with the same expression plasmid minus the CYP2D6 cDNA insert were provided by Dr. S. W. Ellis (Department of Medicine and Pharmacology, University of Sheffield, U.K.). The catalytic and immunologic properties of these microsomes have been described and validated [32].

Determination of K_i Values in Human Liver Microsomes

The incubation conditions were essentially those of Otton *et al.* [8]. Dextromethorphan (0.5, 1.0, or 5.0 μM, final concentration) was used as the CYP2D6 substrate. Dextromethorphan was incubated with an NADPH-generating system (0.1 μmol NADP, 0.2 U glucose-6-phosphate dehydrogenase, 1 μmol glucose-6-phosphate, and 0.5 μmol $MgCl_2$) in 0.2 M phosphate buffer (pH 7.4). At each of the substrate levels, dextrorphan production was monitored in the absence and in the presence of three concentrations of each amphetamine analog. Solutions of amphetamine analogs were prepared freshly in 1.15% KCl. The reaction was started by addition of 75 μg microsomal protein (35 μL, prepared from human liver K12), allowed to proceed at 37° in a shaking water bath for 30 min, and stopped by the addition of 20 μl of 70% perchloric acid and cooling the samples on ice. Internal standard (levallorphan) was added and proteins were sedimented by centrifugation at 700 *g* for 10 min. The supernatant (50 μL) was assayed for dextrorphan by HPLC. Dextrorphan levels in the samples were stable for at least 6 days at room temperature.

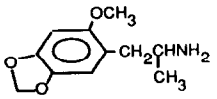
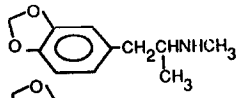
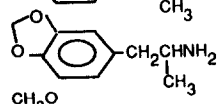
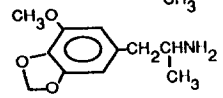
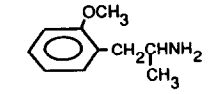
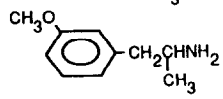
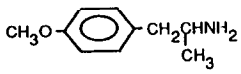
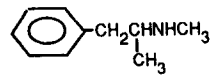
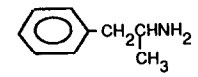
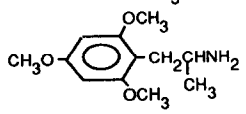
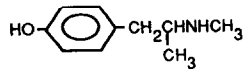
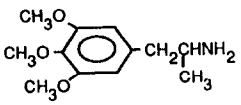
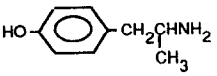
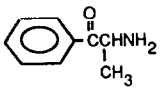
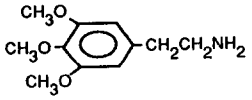
Characterization of PMA O-Demethylation in Human Liver Microsomes

In the kinetic study, PMA (2.5 to 1000 μM) was incubated with microsomes prepared from six EM livers (K12, K14, K20, K21, K23, and K24) and one PM liver (K16). The incubation conditions were as described above. Preliminary studies demonstrated that the production of the metabolite 4-hydroxyamphetamine was linear up to 600 mg/mL microsomal protein and throughout a 30-min incubation. The effect of quinidine (0.001 to 1.6 μM) and quinine (0.02 to 400 μM) on PMA O-demethylation was examined using microsomes prepared from human liver K12 and a PMA concentration of 57.5 μM (K_m value for this liver). The rates of O-demethylation of 5 μM dextromethorphan and 50 μM PMA were compared using microsomes from twelve EM livers and two PM livers (K19 and K16). The incubation time for both reactions was 30 min.

Metabolism and Methoxyamphetamines during Incubation with Yeast Microsomes

Levels of PMA and four other methoxyamphetamines (2-methoxyamphetamine, 3-methoxyamphetamine, 3,4,5-TMA and 2,4,6-TMA) were measured before and after a 20-min incubation with 3 mg microsomal protein from control or recombinant yeast. Cumene hydroperoxide (75

TABLE 1. Chemical structures and apparent K_i values of amphetamine analogs tested

Amphetamine analog	Chemical structure	Apparent K_i (μM)
(*d,l*)-2-Methoxy-4,5-methylendioxyamphetamine (MMDA-2)		0.17 (0.24, 0.1)*
(*d,l*)-3,4-Methylenedioxymethamphetamine (MDMA)		0.6 (1.0, 0.2)
(*d,l*)-3,4-Methyleneioxyamphetamine (MDA)		1.8 (2.8, 0.8)
(*d,l*)-3-Methoxy-4,5-methylenedioxyamphetamine (MMDA)		2.2 (3.0, 1.4)
(*d,l*)-2-Methoxyamphetamine		11.5 (12, 11)
(*d,l*)-3-Methoxyamphetamine		17.5 (15, 20)
(*d,l*)-4-Methoxyamphetamine (PMA)		24 (17.5, 30)
(+)-Methamphetamine		25 (25, 25)
(+)-Amphetamine		26.5 (28, 25)
(*d,l*)-2,4,6-Trimethoxyamphetamine (2,4,6-TMA)		33 (21, 45)
(*d,l*)-4-Hydroxymethamphetamine		60 (50, 70)
(*d,l*)-3,4,5-Trimethoxyamphetamine (3,4,5-TMA)		128 (125, 130)
(+)-4-Hydroxyamphetamine		195 (240, 150)
(*d,l*)-Cathinone		340
(*d,l*)-Mescaline		No interaction

* Values in parentheses are two determinations of K_i measured on different days.

μM) was used instead of NADPH [33]. The reaction volume was 0.5 mL, and the final concentration of the methoxyamphetamines was 1 μM. The reaction was stopped by the addition of 20 μL of 70% perchloric acid. The incubations with microsomes containing recombinant CYP2D6 were repeated with 10 μM quinidine included in the reaction mixtures.

Effect of Microsomal Preincubation with MDMA on CYP2D6 Activity

Microsomes from one EM liver (K12) were incubated with the NADPH-generating system for 20 min at 37°. MDMA (0.5 or 1.5 μM) was added either at the outset of the incubation, or immediately following. Dextromethorphan (final concentration 10 μM) was then added to all samples,

and the incubation proceeded for a further 30 min, and was stopped by the addition of 20 μL of 70% perchloric acid and cooling on ice. The samples were prepared as described above, and assayed for dextrorphan.

HPLC Assays

Dextrorphan concentrations were measured by the HPLC assay described by Broly *et al.* [34], except that the excitation and emission wavelengths were set at 195 and 280 nm, respectively, and a phenyl column (5 μm, 15 × 0.46 cm, Chromatography Sciences Co., Montreal, PQ) was used. The methoxyamphetamines and the PMA metabolite 4-hydroxyamphetamine were assayed by HPLC using fluorescence detection. 2-Methoxyamphetamine was added (as the internal standard) to the samples after the reaction was stopped. To study 2-methoxyamphetamine disappearance, 4-hydroxyamphetamine was used as the internal standard. After centrifugation, 50 μL of the supernatant was injected onto the same column as described above. The mobile phase was 10 mM potassium phosphate buffer (pH 5.0) containing 0.1% triethylamine and acetonitrile (90:10, v/v). The flow rate was 1.0 mL/min. The detector was set at 200 and 280 nm for excitation and emission wavelengths, respectively. The sensitivity of the assay for 4-hydroxyamphetamine was 50 pmol/mL.

Data Analysis

The apparent inhibition constant (K_i) of each amphetamine analog was determined graphically by the method of Dixon and Webb [35]. Non-linear regression analysis of the untransformed data using the computer program PHARM/PCS [36] confirmed the K_i values and showed that the data fitted the equation for competitive inhibition. The kinetic parameters of PMA O-demethylation were calculated by the computer program ENZFITTER [37].

RESULTS

CYP2D6 Inhibition by Amphetamine Analogs

With the exception of mescaline, all the amphetamine analogs tested were competitive inhibitors of microsomal CYP2D6 activity. The apparent K_i values ranged from 0.17 to 340 μM (Table 1). The four amphetamines with a methylenedioxy group, i.e. MMDA-2, MDMA, MDA and MMDA, were the most potent inhibitors of CYP2D6, having K_i values in a nanomolar or low micromolar range. Mono-methoxy substituted amphetamines (at the 2, 3, or 4 position), (+)-amphetamine and (+)-methamphetamine had K_i values in the 12 to 27 μM range. The presence of three methoxy groups (2,4,6-TMA or 3,4,5-TMA) decreased inherent affinity for CYP2D6; these compounds had K_i values of 33 and 128 μM, respectively. 4-Hydroxymethamphetamine and 4-hydroxyamphetamine were several-fold less potent inhibitors of CYP2D6 than methamphetamine and amphetamine themselves. Cathinone, an amphetamine-type drug with a keto group on the alpha carbon, was a very weak inhibitor, having a K_i of 340 μM. Mescaline, which is structurally similar to 3,4,5-TMA but has no methyl group alpha to the nitrogen, did not interact with CYP2D6 at concentrations up to 250 μM. Under these experimental conditions, only competitive-type inhibition was observed.

Oxidation of PMA to 4-Hydroxyamphetamine

Figure 1 compares the rate of formation of 4-hydroxyamphetamine from PMA incubated with microsomes from one human liver (K12) and one PM (K16) liver. Only trace amounts of 4-hydroxyamphetamine were detected at high substrate concentrations (more than 100 μM PMA) in incubations with the PM liver microsomes. The Michaelis–Menten parameters of this O-demethylation reaction in microsomes from six EM livers are given in Table 2. The mean (±SD) K_m was 59.2 ± 22.4 μM and the V_{max} was 29.3 ± 16.6 nmol/mg/hr. PMA O-demethylation was inhibited stereoselectively by the quinidine/quinine enantiomer pair (IC_{50} value = 0.09 μM for quinidine vs 30 μM for quinine). Furthermore, the rates of O-demethylation of PMA and dextromethorphan were highly correlated in microsomes from fourteen human livers (Fig. 2). The correlation coefficient was $r = 0.975$ ($P < 0.001$).

Methoxyamphetamines as Substrates of CYP2D6

More than 95% of the five methoxyamphetamines were recovered unchanged after incubations with control yeast microsomes (Fig. 3). However, the same figure shows that the recovery after incubation with recombinant yeast microsomes varied between 80% for PMA and 4% for 3,4,5-TMA. The recovery of all five compounds was greater than 85% when 10 μM quinidine was included in the incubations with recombinant CYP2D6.

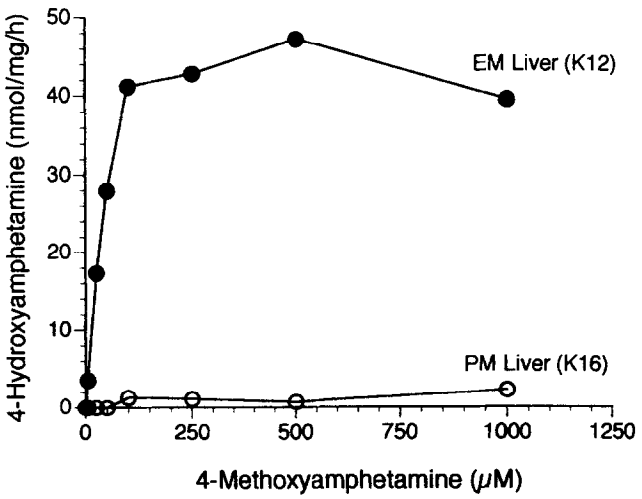


FIG. 1. 4-Hydroxyamphetamine formation in incubations of PMA with EM and PM liver microsomes. Each point on the graph represents the mean of duplicate incubations.

TABLE 2. Apparent kinetic parameters of PMA O-demethylation in microsomes from 6 EM human livers

Liver	K_m (μM)	V_{max} (nmol/mg/hr)
K12	57.5	51.5
K20	46.1	48.0
K21	99.2	27.6
K14	68.9	19.6
K24	38.4	15.0
K23	45.1	14.2
Mean ± SD	59.2 ± 22.4	29.3 ± 16.6

Effect of Preincubation of MDMA on Microsomal CYP2D6 Activity

Preincubation of MDMA with liver microsomes in the presence of an NADPH-generating system for 20 min markedly enhanced the inhibition of CYP2D6 activity by MDMA, as assessed by the rate of dextromethorphan O-demethylation (Fig. 4). Preincubation with 1.5 μM MDMA decreased CYP2D6 activity to about 7% of the control level, compared to 76% in samples preincubated without MDMA. The enhanced inhibitory effect of MDMA after preincubation was NADPH dependent (data not shown) and related to the MDMA concentration.

DISCUSSION

All the amphetamine analogs sharing the phenylisopropylamine structure were inhibitors of CYP2D6 activity. Their apparent K_i values ranged over three orders of magnitude—from 0.17 to 340 μM. The results listed in Table 1 suggest that lipophilicity and certain substituent groups within this series play a role in the binding affinity to CYP2D6 enzyme. The polar metabolite of amphetamine, (+)-4-hydroxyamphetamine, was 7-fold less potent than (+)-amphetamine itself (K_i 195 vs 26.5 μM), and the metabolite of methamphetamine, 4-hydroxymethamphetamine, was 2.5-fold less potent than methamphetamine (K_i 60 vs 25 μM). Mescaline lacks the β-methyl group of 3,4,5-TMA, making it less lipophilic as judged by its lower octanol:buffer partition coefficient [38]. It did not interact with microsomal CYP2D6 at the concentrations examined here, whereas 3,4,5-TMA was a weak inhibitor (K_i 128 μM). The presence of a methylenedioxy group in the 3,4-positions of both amphetamine and methamphetamine increased the affinity for CYP2D6 considerably (a 15-fold increase for amphetamine, from 26.5 to 1.8 μM, and a 42-fold increase for methamphetamine, from 25 to 0.5 μM). Addition of a methoxy group to the 2- or 3-position of amphetamine also increased the affinity for CYP2D6. A methoxy group in the 2-position was the more effective, increasing the affinity 2-fold (from 26.5 to 11.5 μM). A methoxy group in the 4-position had little effect on the affinity of amphetamine for CYP2D6, as indicated by the similar K_i values of PMA and amphetamine (24 and 26.5 μM, respectively). The compound with the highest affinity for CYP2D6 was the amphetamine analog MMDA-2, which has both a methoxy group in the 2-position and a methylenedioxy group (K_i 0.17 μM).

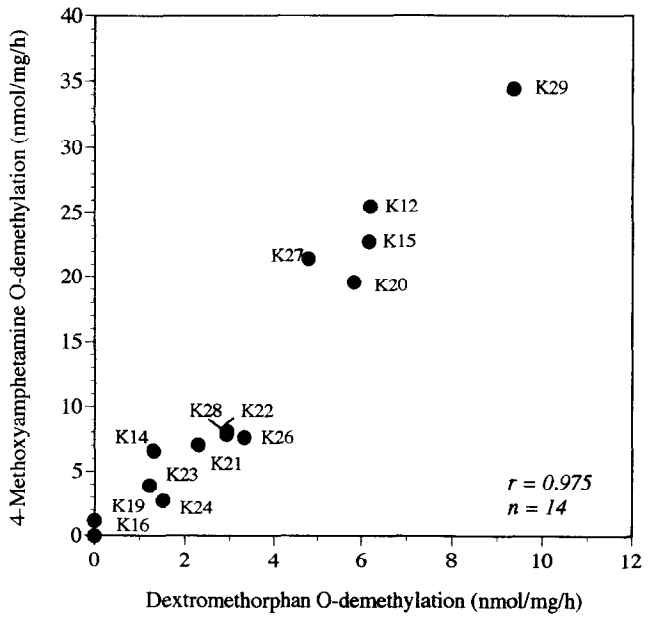


FIG. 2. Comparison of PMA O-demethylation with dextromethorphan O-demethylation. Microsomes from fourteen human livers were incubated with PMA (80 μM) or dextromethorphan (5 μM). Data are expressed as the means of duplicate incubations.

Our *in vitro* PMA oxidation results confirm the clinical observation that a subject with deficient CYP2D6 activity had impaired capacity to O-demethylate this drug [11]. The *in vitro* evidence for PMA as a substrate of CYP2D6 is 3-fold. First, the O-demethylation of PMA was deficient in microsomes of two PM livers. Second, PMA O-demethylation was stereoselectively inhibited by the quinidine/quinine enantiomer pair, a characteristic of CYP2D6 catalyzed reactions [39, 40]. Third, in microsomes from fourteen human livers, PMA O-demethylation was highly correlated ($r = 0.975$, $P < 0.001$) with dextromethorphan O-demethylation, a prototypic reaction of CYP2D6. The clinical response to PMA is reported to be highly variable, and PMA abuse was associated with several unexpected deaths of young people over a short period of 5 months in 1973 [14, 15]. The mechanism(s) of its toxicity is unknown, but it has been speculated that accumulation of the drug in PMs may have contributed to these fatalities [11].

The preliminary studies looking at disappearance of PMA and four other illicit methoxyamphetamine analogs indicated that all were substrates of the recombinant CYP2D6 enzyme. Based on these results, PMA was metabolized at the slowest rate, and 3,4,5-TMA was consumed almost completely during the 20-min incubation (only 4% of the drug added was recovered after incubation). This observation is difficult to reconcile with the relatively high K_i value of 3,4,5-TMA (K_i 128 μM) estimated using dextromethorphan O-demethylase activity in human liver microsomes. One explanation for this observation is that during the inhibition studies, 3,4,5-TMA is rapidly metab-

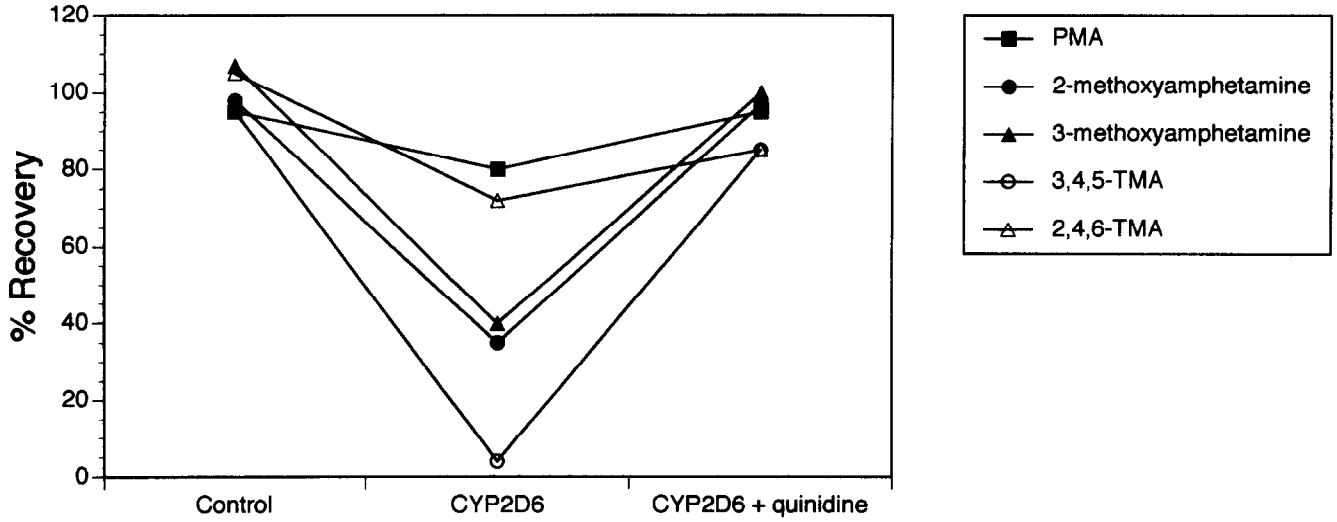


FIG. 3. Percent recovery of five methoxyamphetamine analogs from incubations with yeast microsomes. Each drug (1 μM) was incubated with 3 mg microsomal protein from control yeast (no CYP2D6 cDNA insert) or yeast expressing CYP2D6. The effect of quinidine (10 μM) is also shown.

olized, so that the measured K_i value is an over-estimate. The effectiveness of quinidine in blocking the rapid disappearance of 3,4,5-TMA (Fig. 3) argues that the disappearance is related to the activity of the recombinant CYP2D6, not to chemical instability of 3,4,5-TMA during the incubation.

The K_i of MDMA observed here (0.6 μM) is similar to the K_m of MDMA demethylenation (1.7 μM) observed by Tucker and colleagues [16] using an expressed form of CYP2D6. Similarly low K_m values characterized this reaction in rat liver microsomes [41]. Substrate inhibition at high MDMA concentrations (>1 mM) was observed previously [16]. Our study demonstrated that preincubation of MDMA with human liver microsomes and NADPH significantly enhanced its inhibition of CYP2D6 activity (Fig. 4), suggesting mechanism-based inhibition. MDMA, MDA, and other compounds with a methylenedioxy group are known to form inhibitory metabolite complexes with rat liver P450s [42, 43]. The present experiment did not prove that the inhibitory metabolite is formed via CYP2D6; it may arise from some other liver microsomal enzyme. Although the *in vivo* significance of the metabolite complex formation by MDMA remains to be established, it is conceivable that the importance of MDMA metabolism by

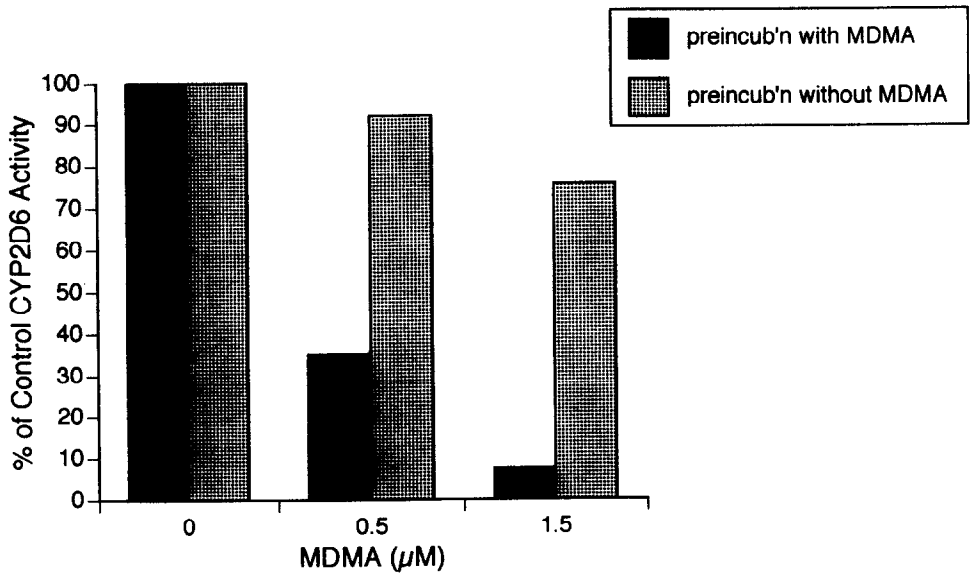


FIG. 4. Effect of preincubation of MDMA inhibition of human microsomal CYP2D6. Human liver microsomes (K12) and an NADPH-generating system were preincubated either with MDMA (filled bars) or without MDMA (dotted bars) for 15 min. Thereafter, CYP2D6 activity was measured by dextrorphan formed during an additional 30-min incubation with 5 μM dextromethorphan. Data are expressed as the means of duplicate incubations. Control velocities were 2.47 and 3.34 nmol dextrorphan formed · mg protein^{-1} · min^{-1} for incubations with and without MDMA, respectively.

CYP2D6 is limited by the "suicide" substrate effect. It is also possible that this mechanism of inhibition by MDMA may cause long-lasting drug interactions with other CYP2D6 substrates.

In conclusion, data presented here demonstrate that most of the phenylisopropylamines tested interact with human liver CYP2D6 as moderately potent competitive inhibitors. Methylenedioxyamphetamines were highly potent inhibitors of CYP2D6 activity, and in the case of MDMA, metabolite–CYP2D6 complex formation may occur. Use of methylenedioxyamphetamines, i.e. MDMA, may cause drug interactions with other medications that are CYP2D6 substrates. A number of the compounds tested here are known CYP2D6 substrates, but the significance of polymorphic CYP2D6 activity to the abuse liability and toxicity of those drugs is currently unknown.

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