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Short Communication

MECHANISM-BASED INACTIVATION OF CYP2D6 BY METHYLENEDIOXYMETHAMPHETAMINE

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ABSTRACT:

The potency of methylenedioxymethamphetamine (MDMA) as a mechanism-based inhibitor of CYP2D6 has been defined using microsomes prepared from yeast expressing the enzyme and from three human livers. The inhibitory effect was increased by preincubation through formation of a metabolic intermediate complex. Inactivation parameters (k_{inact} and K_I), defined with respect to the *O*-demethylation of dextromethorphan, were 0.29 ± 0.03 (S.E.) min^{-1} and 12.9 ± 3.6 (S.E.) μM for yeast-expressed CYP2D6, and 0.26 ± 0.02 min^{-1} and 14.4 ± 2.5 μM, 0.15 ± 0.01 min^{-1} and 8.8 ± 2.6 μM, and 0.12 ± 0.05 min^{-1} and 45.3 ± 32.1 μM for the liver microsomal preparations. The rate of inactivation of CYP2D6 by MDMA decreased when quinidine, a competitive inhibitor of CYP2D6, was added to the primary incubation mixture. However, inactivation was unaffected by the addition of glutathione. The results indicate that MDMA is a potent mechanism-based inhibitor of CYP2D6, with implications for understanding its in vivo disposition and drug interaction potential.

MDMA (commonly known as "Ecstasy") is a synthetic amphetamine derivative that has become a popular recreational drug, particularly in the "rave" culture (Cole and Sumnall, 2003). Case reports of exaggerated responses and death associated with its use suggest that some individuals are at an increased risk of toxicity (Tucker et al., 1994). Hyperthermia, tachycardia, convulsions, rhabdomyolysis, and acute liver and renal failure following MDMA use in nightclubs are well documented, and longer-term use is linked to irreversible changes in serotoninergic function (Ramamoorthy et al., 2002). Although administration of MDMA and its metabolite methylenedioxyamphetamine to rats produces serotonergic nerve terminal degeneration, these compounds are not neurotoxic when injected directly into the brain. This suggests a requirement for their peripheral metabolism to neurotoxic metabolites (Easton et al., 2003) which, presumably, are then actively transported into the brain. These metabolites are believed to be downstream products formed after the opening of the methylendioxyphenyl ring, a process which is mainly mediated by CYP2D6 as a high-affinity enzyme (Tucker et al., 1994; Kreth et al., 2000), with low-affinity contributions from CYP1A2, CYP2B6, and CYP3A4 (Kreth et al., 2000).

CYP2D6 is a polymorphic enzyme and a functional form is absent in 5 to 9% of Caucasians as a result of autosomal recessive inheritance of gene mutations (Zanger et al., 2004). Thus, it has been suggested that genetically deficient metabolism of MDMA may help to explain why some users of "Ecstasy" appear to be more sensitive to its acute effects (Tucker et al., 1994; Lin et al., 1997). However, recent studies have found no obvious link between inherited *CYP2D6* deficiency and MDMA intoxication (O'Donohoe et al., 1998; Schwab et al., 1999; Gilhooly and Daly, 2002). Wu et al. (1997) reported that MDMA is a potent competitive inhibitor of CYP2D6 in human liver microsomes. In addition, they showed that the inhibitory effect of MDMA was increased following preincubation with human liver microsomes in the presence of NADPH. Later studies (Delaforge et al., 1999) confirmed that MDMA forms a metabolic intermediate complex (MIC) with human CYP2D6 expressed in yeast microsomes. However, a quantitative definition of the potency of MDMA as a mechanism-based inhibitor of CYP2D6 is lacking. Thus, the kinetics of the inhibitory effects of MDMA on CYP2D6 in human liver microsomes from extensive metabolizer (EM) subjects and microsomes prepared from yeast expressing the human enzyme were investigated in this study.

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Materials and Methods

Drugs and Chemicals. Racemic MDMA was provided by Professor R. Forrest (Department of Forensic Pathology, Royal Hallamshire Hospital, Sheffield, UK). Dextrorphan tartrate was obtained from Sigma/RBI (Natick, MA). Quinidine, dextromethorphan hydrobromide, and glutathione were purchased from Sigma Chemical (Poole, Dorset, UK). Laudanosine was obtained from Aldrich Chemical Co. (Poole, Dorset, UK). Glucose 6-phosphate (G6P) dehydrogenase (grade II) and the disodium salts of G6P and NADP were purchased from Roche Diagnostics (Lewes, UK). Acetonitrile and orthophosphoric acid were obtained from Fisher Scientific Co. (Loughborough, UK). Triethylamine was purchased from BDH (Poole, Dorset, UK).

Preparation of Microsomes from Human Liver and Yeast Expressing CYP2D6. Three human liver samples (HL6, HL7, and HL17), genotyped as being from EMs of CYP2D6, were used. These samples had been acquired with the approval of the South Sheffield Research Ethics Committee and the local coroner. Clinical details of the donors are described elsewhere (Crewe et al., 1997). Human liver microsomes were prepared as described previously (Otton et al., 1988) and stored at −80°C as a suspension in 0.1 M potassium phosphate buffer (pH 7.4) containing 30% (v/v) glycerol. Microsomal protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The heterologous expression of human CYP2D6 in

ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; G6P, glucose 6-phosphate; MIC, metabolic intermediate complex; EM, extensive metabolizer with respect to CYP2D6; P450, cytochrome P450; HPLC, high-performance liquid chromatography.

the yeast *Saccharomyces cerevisiae* and the preparation of yeast microsomes have been described previously (Ellis et al., 1992).

Time-Dependent Inhibition of CYP2D6 Activity. The effect of 5 min of preincubation with MDMA on the inhibition of dextromethorphan *O*-demethylation was investigated. All incubations were carried out at 37°C in a shaking water bath. The preincubation tubes contained MDMA (0, 0.625, 1.25, 2.5, 6.25, and 12.5 μM, final concentrations) in 50 μl of KCl (1.15%, w/v) and a NADPH-generating system (0.4 μmol of NADP, 4 μmol of G6P, 2 μmol of $MgCl_2$, and 0.4 unit of G6P-dehydrogenase) in 100 μl of 0.2 M potassium phosphate buffer (pH 7.4). The reactions were started by the addition of 50 μl of yeast microsomes expressing CYP2D6 (20 pmol of P450). After 5 min, 50 μl of dextromethorphan solution was added to the preincubation mixture (final concentration 20 μM) to assess the remaining CYP2D6 activity. Incubations were carried out in duplicate for another 5 min and terminated by the addition of 25 μl of perchloric acid (60%, v/v). Coincubations in which MDMA and dextromethorphan were added together at the end of the 5-min preincubation were also carried out (MDMA was not added to the preincubation tubes in these experiments).

The extent of CYP2D6 inactivation during the coincubation period, in addition to that occurring during preincubation, was assessed by determining the V_{max} of dextromethorphan in the absence and presence of MDMA. Thus, the above experiments were repeated using a range of dextromethorphan concentrations (0–200 μM) with and without MDMA (final concentration 5 μM). Incubation mixtures comprised 50 μl of yeast microsomal suspension (20 pmol of P450), MDMA and dextromethorphan, each dissolved in 50 μl of KCl (1.15, w/v), and 100 μl of the NADPH-generating system dissolved in 0.2 M potassium phosphate buffer (pH 7.4). All incubations were carried out in duplicate at each substrate concentration, and each experiment was repeated twice.

The Effect of Preincubation Time on the Inactivation of CYP2D6. Incubation tubes contained MDMA at final concentrations of 0, 2, 4, 8, 20, and 40 μM in 125 μl of KCl (1.15%, w/v) and a NADPH-generating system (2 μmol of NADP, 20 μmol of G6P, 9.99 μmol of $MgCl_2$, and 6 units of G6P-dehydrogenase) in 250 μl of 0.2 M potassium phosphate buffer (pH 7.4). The reactions were started by the addition of 125 μl of yeast microsomes expressing CYP2D6 (160 pmol of P450). Aliquots (62 μl) were removed at 0, 1, 2, 3, 4, and 5 min from the preincubation mixture and added to other incubation tubes. Each of these tubes contained 50 μl of dextromethorphan solution (final concentration 20 μM) and 69 μl of KCl. The incubation procedure (carried out in duplicate) and the assessment of remaining CYP2D6 activity were as described above.

These experiments were repeated using microsomes prepared from each of the three human livers. Preincubation times ranged from 0 to 30 min, and incubation was carried out for 10 min. The preincubation reactions were started by the addition of 125 μl of human liver microsomal suspension (1 mg of protein). All other incubation conditions were as described for the experiments involving yeast microsomes.

Spectral Complex Formation. Microsomes from yeast expressing CYP2D6 (500 pmol of P450) were mixed with NADPH (4 mM) in 0.2 M phosphate buffer (pH 7.4) to a final volume of 1 ml. The mixture was divided between two 0.5-ml cuvettes of 1 cm path length. MDMA solution was added to the sample cuvette to give a final concentration of 800 μM (lower concentrations were evaluated but did not produce measurable complex). An equivalent volume of water was added to the reference cuvette. Difference spectra were recorded at room temperature using a double-beam UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan) scanning between 500 and 380 nm at 0, 2, 5, 15, and 30 min.

Effects of Quinidine and Glutathione on the Inactivation of CYP2D6. Yeast microsomes (20 pmol of P450) were preincubated for 5 min at 37°C with the NADPH-generating system in the presence of quinidine (0.001, 0.01, 0.1, or 1 μM) or glutathione (0.1, 1, 5, or 10 μM) with or without MDMA (5 μM). The samples were then assayed for remaining CYP2D6 activity following addition of dextromethorphan (20 μM).

Nonspecific Microsomal Binding of MDMA. Nonspecific binding of MDMA to yeast microsomes was measured by ultracentrifugation using Millipore Centrifree Micropartition devices (Millipore Corporation, Stonehouse, UK).

Aliquots (500 μl) of inactive microsomes from yeast expressing CYP2D6 (final protein concentration 1.27 mg/ml) were spiked with different concentrations of MDMA (0.5–2.2 μM) and 50 μl of potassium phosphate buffer (pH 7.4), incubated in the ultrafiltration reservoir at 37°C for 10 min, and then centrifuged at 1000*g* for 10 min. The ultrafiltrates were assayed for MDMA by HPLC. Control experiments, without the addition of microsomal protein, were carried out to assess the binding of MDMA to different components of the ultrafiltration device. The extent of this binding was calculated using the following equation (adapted from Ballard, 1998):

$$fu_{mic} = \frac{1}{1 + \left[\frac{C \times fu_{mem} \times fu_c}{Cu_c} - \frac{1}{fu_R}\right]}$$

where fu_{mic} is the fraction of unbound drug in microsomes, C is the total drug concentration in the reservoir (microsomal samples), fu_{mem} is the fraction of drug not bound to the membrane (calculated using fu_c and fu_R and measured concentrations after centrifugation of buffer samples), fu_c is the fraction of drug not bound to the receiving cup (calculated using buffer samples placed in the cup), Cu_c is the concentration of drug in the cup (after centrifugation of microsomal samples), and fu_R is the fraction of drug not bound to the reservoir (calculated using buffer samples placed in the reservoir).

Drug Assays. Dextrorphan was assayed using modifications of the methods described by Jacqz-Aigrain et al. (1989, 1993). Laudanosine (15 μl, 5 μg/ml) was added as the internal standard to all incubation samples, which were then vortexed and centrifuged at 13,000 rpm (10,000*g*) for 10 min. The supernatant (50 μl) was injected onto an HPLC system fitted with a radial compression column containing NovaPak CN reversed phase material (Waters, Milford, MA) and a CN precolumn. The mobile phase consisted of acetonitrile/water (10:90, v/v) containing 0.03% (v/v) triethylamine adjusted to pH 3.1 with orthophosphoric acid. A model 2080 HPLC pump (Jasco, Tokyo, Japan) was used for solvent delivery at a flow rate of 3 ml min^{-1}. The eluate was monitored with an LC240 fluorescence spectrophotometric detector (PerkinElmer UK, Beaconsfield, Buckinghamshire, UK) set at excitation and emission wavelengths of 270 and 312 nm, respectively. The retention time of dextrorphan was 6.2 min, and the intra- and interday coefficients of variation of the assay (concentration range 0.1–0.8 μM) were 1.8% and 4.4%, respectively.

MDMA was measured using the same HPLC system and conditions (retention time 3 min). The intra- and interday coefficients of variation of the assay (concentration range 1–2 μM) were 6% and 8%, respectively.

Data Analysis. IC_{50}, V_{max}, and K_m values were calculated by nonlinear least-squares regression using GraFit (Version 5.0.4; Erithacus Sofware, Horley, Surrey, UK). Inactivation constants (k_{inact} and K_I) were estimated as described by Silverman (1995). Any loss of enzyme activity unrelated to the inhibitor was accounted for by expressing the control rate of CYP2D6-mediated metabolism (*O*-demethylation of dextromethorphan to dextrorphan) in yeast and human liver microsomes as a percentage of that in time-matched control samples without inhibitor. The rate constant for initial inactivation (k_{obs}) at each inhibitor concentration was estimated from a plot of the slope of the natural log of "percent remaining activity" versus preincubation time. Values of k_{inact} and K_I were obtained from a double reciprocal plot of k_{obs} versus the corresponding inhibitor concentrations. These values were used as initial estimates in solving the following equation with GraFit:

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]}$$

where $[I]$ is the initial inhibitor concentration, k_{inact} is the maximum rate constant for inactivation when $[I]$ approximates infinity, and K_I is the inhibitor concentration that produces half the maximal rate of inactivation. Comparisons between estimated kinetic constants were made using the Z test implemented in Excel.

Results

The inhibitory effect of MDMA on dextromethorphan *O*-demethylation in yeast microsomes expressing CYP2D6 was increased by preincubation. The IC_{50} values for inhibition were 9.4 ± 2.1 (S.E.)

μM and 5.0 ± 0.7 (S.E.) μM for coincubation and preincubation, respectively. Dextromethorphan *O*-demethylation was inhibited in a time- and concentration-dependent manner by MDMA using microsomes prepared from yeast expressing CYP2D6 (Fig. 1) and human liver microsomes (data not shown). The percentage disappearance of MDMA (2 μM) observed at the end of the preincubation step was 56% for yeast (5 min) and 44%, 18%, and 10% (30 min) for HL6, HL7, and HL17, respectively. Inactivation parameters (k_{inact} and K_I) determined for CYP2D6 in yeast microsomes (Fig. 1) were 0.29 ± 0.03 (S.E.) min^{-1} and 12.9 ± 3.6 (S.E.) μM, respectively. Corresponding values for the three human liver samples were 0.26 ± 0.02 min^{-1} and 14.4 ± 2.5 μM (HLM6), 0.15 ± 0.01 min^{-1} and 8.8 ± 2.6 μM (HLM7), and 0.12 ± 0.05 min^{-1} and 45.3 ± 32.1 μM (HLM17).

As shown in Fig. 2, the maximal rate of dextrorphan formation (V_{max}) by yeast microsomes expressing CYP2D6 was unaffected by the presence of coincubated MDMA (5 μM). V_{max} values in the absence and presence of MDMA were similar [1.05 ± 0.02 (S.E.) and 1.07 ± 0.03 (S.E.) pmol/min/pmol P450, respectively; $p = 0.49$; *Z* test]. However, corresponding K_m values (4.99 ± 0.52 μM versus 9.23 ± 1.06 μM) were significantly different ($p < 0.001$; *Z* test), indicating some degree of non-mechanism-based inhibition. Assuming that the shift in K_m corresponded to competitive inhibition with an extent of $1 + [I]/K_i$, and knowing the MDMA concentration in the incubation mixture (5 μM), a value for the competitive inhibition constant (K_i) of 5.9 μM was estimated. Because no significant nonspecific microsomal binding of MDMA was observed, no correction of this K_i value was necessary. Spectral difference scanning of yeast microsomes during incubation with MDMA in the presence of a NADPH-generating system indicated a time-dependent increase in absorbance at 456 nm (Fig. 3).

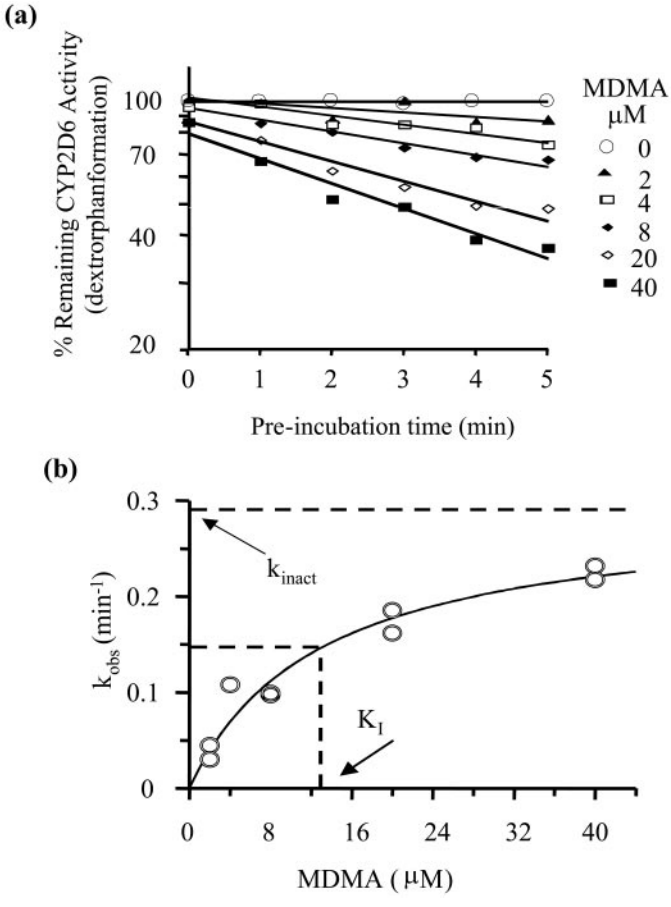

FIG. 1. Inactivation of CYP2D6 by MDMA in yeast microsomes expressing CYP2D6. a, relationship between preincubation time with various concentrations of MDMA and CYP2D6 activity as measured by percentage of remaining activity of dextrorphan formation compared with control. b, plot of inactivation rate constant as a function of MDMA concentration used to determine k_{inact} and K_I values. The data points at each concentration refer to duplicate experiments and the solid line is the best fit obtained by nonlinear least-squares regression.

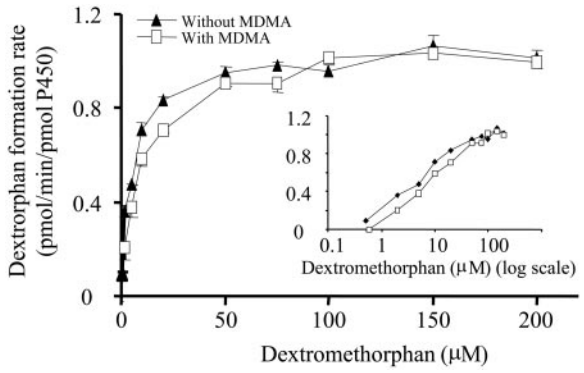

FIG. 2. The effect of coincubation with MDMA on dextrorphan formation rate in yeast microsomes expressing CYP2D6. Increasing concentrations of dextromethorphan were associated with no change in the maximum rate of dextrorphan formation. The inset shows a shift of the curve to the right during coincubation of dextromethorphan with MDMA.

The rate of inactivation by MDMA decreased when quinidine, a competitive inhibitor of CYP2D6, was added to the primary incubation mixture containing yeast microsomes (Fig. 4a). Inactivation decreased with increasing concentrations of quinidine. Figure 4b shows that the addition of glutathione had little effect on the rate of inactivation of CYP2D6 by MDMA in yeast microsomes.

Discussion

The findings confirm that MDMA is a mechanism-based inhibitor of CYP2D6. Preincubation of MDMA in the presence of a NADPH-generating system caused an increase in the inhibitory effect of the drug, and the rate of inactivation of CYP2D6 was time- and concentration-dependent. The inactivation was associated with a time-dependent increase in absorbance at 456 nm during the preincubation period, supporting the formation of a MIC. The decrease in inactivation observed in the presence of the competitive CYP2D6 inhibitor quinidine suggests that transformation of MDMA to the MIC occurs at the active site of CYP2D6. The lack of effect of glutathione on the inactivation of CYP2D6 suggests that, once the reactive intermediate has been generated, it does not leave the active site. These findings are consistent with criteria for a mechanism-based inhibitor (Silverman, 1995).

The presence of the methylenedioxyphenyl ring in MDMA and methylenedioxyamphetamine is the most likely reason for formation of a MIC with CYP2D6, although such complexes are also formed by amine groups. P450-mediated oxidation of the methylene carbon in both compounds would lead to formation of unstable intermediates, which are then demethylenated to catechols (3,4-dihydroxymethamphetamine and α-methyldopamine) or dehydrated to carbenes. The former compounds may be oxidized further to orthoquinones, which can react with nucleophilic groups on macromolecules or conjugate with glutathione to form neurotoxins (Easton et al., 2003). The carbene intermediates are likely to form covalent complexes with the heme iron of CYP2D6, yielding the characteristic type 3 spectrum (Ortiz de Montellano, 1995).

Values of k_{inact} and K_I were generally similar for yeast microsomes and microsomes from the three livers investigated. The differences

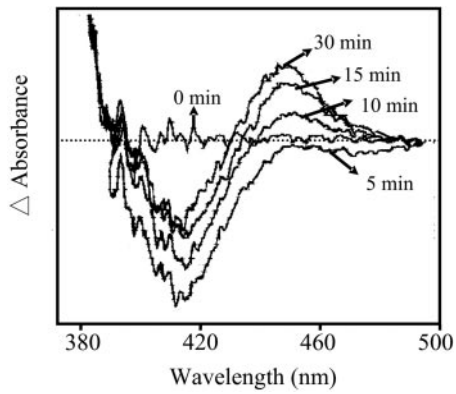

FIG. 3. Difference spectrum indicating the formation of a MIC during incubation of MDMA (800 μM) with yeast microsomes expressing CYP2D6.

between the livers are likely to be due to genotypic differences affecting specific activity. The liver samples used in the present study were genotyped as being *1*1, *1*4, and *1*2 for HL6, HL7, and HL17, respectively. It has been shown that livers with the CYP2D6*1/*2 genotype exhibit 20 to 40% lower intrinsic clearances with respect to MDMA and dextromethorphan than the mean value associated with wild-type livers (*1/*1) (Ramamoorthy et al., 2002). It is likely that MDMA at higher concentrations also inactivates CYP 1A2, 2B6 and 3A4, inasmuch as these enzymes contribute to low-affinity *N*-demethylenation of the compound (Kreth et al., 2000).

The values of k_{inact} (0.12–0.26 min^{-1}) and K_I (8.8–45.3 μM) for MDMA are similar to those reported for paroxetine (k_{inact} = 0.25 min^{-1}, K_I = 6.6 μM), another mechanism-based inhibitor of CYP2D6 that also has a methylendioxyphenyl ring (Bertelsen et al., 2003). Thus, the impact of mechanism-based inactivation of its own metabolism by MDMA in vivo is expected to be similar to that observed with comparable doses of paroxetine; that is, dose- and concentration-dependent kinetics, persistent inactivation of CYP2D6, phenocopying of EM individuals to apparent poor metabolizers, and higher exposure to coadministered CYP2D6 substrates (Alfaro et al., 1999; Laine et al., 2001, 2004; Belle et al., 2002; Liston et al., 2002).

The experiments involving coincubation of MDMA and dextromethorphan indicated that further loss of CYP2D6 activity did not occur during the incubation step at higher dextromethorphan concentrations. Observed V_{max} (1.05 pmol/min/pmol P450) and K_m (5.0 μM) values for dextrorphan formation in the absence of MDMA were consistent with previous estimates obtained using baculovirus-transformed insect cells (1.68 pmol/min^{-1} $pmol^{-1}$ and 4.0 μM) (Ramamoorthy et al., 2002) and B-lymphoblastoid cells (10.8 pmol/min^{-1} $pmol^{-1}$ and 5.2 μM) (Kawashiro et al., 1998). There was a small shift in activity in the presence of MDMA, which suggests that the contribution of competitive inhibition of CYP2D6 is small, since the K_i value is greater than 1 (values below 1 are commonly regarded as being associated with potent inhibition). The calculation of K_i assumed no contribution from mechanism-based inactivation to the observed shift in activity. However, inspection of the inset in Fig. 2 indicates that when dextromethorphan concentrations were low, the shift in activity was larger than that observed at higher concentrations. This indicates some contribution of mechanism-based inactivation to the decrease in CYP2D6 activity. Thus, the calculated K_i value may overestimate the potency of competitive inhibition of CYP2D6 by MDMA. Kinetic models that account for simultaneous competitive and mechanism-based inhibition are necessary to define the contribution of each type of inhibition during coincubation.

Recreational doses of MDMA are associated with maximum plasma drug concentrations of around 3 μM (de la Torre et al., 2000). Considering the range of K_I values obtained in the present study (8.8–46.1 μM), it would appear that mechanism-based inactivation of CYP2D6 by MDMA in vivo is unlikely. However, assuming an absorption rate constant of 2.2 h^{-1} (estimated from the data of de la Torre et al., 2000) and a liver blood flow of 90 l/h, the maximum inlet concentration of MDMA to the liver following a 100-mg oral dose could be as high as 22 μM. Accordingly, it is likely that MDMA concentrations at the active site of CYP2D6 will exceed its inactivation K_I during "first pass" through the liver, leading to a marked decrease in enzyme activity in vivo. Indeed, multiple-dose studies of MDMA in humans have demonstrated both concentration- and time-dependent kinetics (de la Torre et al., 2000). The effect of mechanism-based inhibition of CYP2D6 in vivo is a function of the inactivation parameters of the drug (K_I, k_{inact}, and partition ratio) and the synthesis rate of de novo enzyme (Mayhew et al., 2000). The in vitro rate of CYP2D6 inactivation by MDMA is high relative to the observed rate of CYP2D6 loss in cultured precision-cut human liver slices (Renwick et al., 2000). Therefore, under basal conditions, it is likely that inactivation of CYP2D6 exceeds substantially the rate of synthesis of new enzyme. Rapid inactivation of CYP2D6 may result in the phenocopying of extensive metabolizers to poor metabolizers in vivo. This expectation is supported by unpublished data (R. de la Torre, personal communication) showing that, despite the importance of CYP2D6 as the major contributor to the metabolism of MDMA in vitro, there was a relatively small difference in the oral clearance of the drug between a group of EMs and a poor metabolizer subject. The decreased contribution of CYP2D6 to MDMA clearance in vivo, as a consequence of autoinhibition via mechanism-based inactivation, is also consistent with a relatively small inhibitory effect (30%) of paroxetine on MDMA clearance in vivo (Segura et al., 2004).

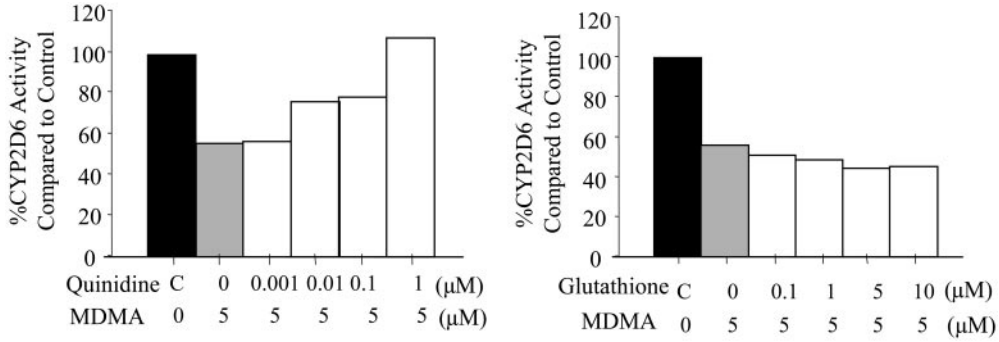

FIG. 4. Effects of the competitive inhibitor quinidine (left panel) and the nucleophile glutathione (right panel) on MDMA-mediated inactivation of CYP2D6 in yeast microsomes. C signifies control experiments with quinidine or glutathione at the same concentrations as those with added MDMA.

In conclusion, the kinetics of inactivation of CYP2D6 by MDMA has been characterized, showing that it is extremely potent in this respect. The full implications of this finding for the in vivo kinetics of the drug, the identification of risk factors for acute and chronic toxicity, and drug-drug interactions remain to be elucidated.

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