Differential Roles of Phase I and Phase II Enzymes in 3,4-Methylenedioxymethamphetamine-Induced Cytotoxicity

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ABSTRACT:
Metabolism plays an important role in the toxic effects caused by 3,4-methylenedioxymethamphetamine (MDMA). Most research has focused on the involvement of CYP2D6 enzyme in MDMA bioactivation, and less is known about the contribution of other cytochrome P450 (P450) enzymes as well as phase II metabolism. In this study, we researched the differential roles of phase I P450 enzymes CYP1A2, CYP3A4, and CYP2D6 and phase II enzymes glutathione S-transferase (GST) and catechol-O-methyltransferase (COMT) on the toxic potential of MDMA. MDMA acts as inhibitor of its own metabolism with a relative potency of inhibition of CYP2D6 > CYP3A4 > CYP1A2 in rat liver microsomes and in human liver [immortalized human liver epithelial cells (THLE)] cells transfected with individual CYP1A2, CYP3A4, or CYP2D6. Cytotoxicity measurements [by 3,4-dihydroxy-5-nitrobenzophenone (Ro-41-0960) and GST by buthionine sulfoximine] showed that CYP2D6 inhibition by quinidine. Inhibition of COMT by 2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone (Ro-41-0960) and GST by buthionine sulfoximine showed that COMT is mainly involved in detoxification of CYP2D6-formed MDMA metabolites, whereas glutathione (GSH) is mainly involved in detoxification of CYP3A4-formed MDMA metabolites. Liquid chromatography/tandem mass spectrometry analyses of MDMA metabolites in the THLE cell culture media confirmed formation of the specific MDMA metabolites and corroborated the observed cytotoxicity. Our data suggest that CYP2D6 and CYP3A4 play an important role in MDMA bioactivation. In addition, further studies are needed to address the differential roles of CYP3A4 and GSH/GST in MDMA bioactivation and detoxification.

3,4-Methylenedioxymethamphetamine [(MDMA) “ecstasy”] is an illicit drug of abuse that possesses stimulant and hallucinogenic properties. Chemically, it is a ring-substituted phenylamine derivative that became widely available in Europe at the beginning of the 1990s. The use of MDMA is mainly associated with nightclubs, dance music, and some subcultures, and a significantly higher level of use is being reported among young people (EMCDDA, 2008).

Consumption of MDMA can lead to severe acute clinical effects such as tachycardia, hypertension, hyperthermia, intracranial hemorrhage, serotonin syndrome, and even death. Furthermore, reported chronic effects have been related to disruption of the serotonergic function (Parrott 2001; Garcia-Repetto et al., 2003; Schifano 2004; de la Torre et al., 2004; Carro et al., 2006). Cases of hepatotoxicity due to MDMA ingestion have also been reported, yet it is probable that many more cases are subclinical and remain undetected. Subacute hepatotoxicity can ultimately lead to severe liver damage after repeated exposure, and MDMA is considered to be a cause of drug-induced liver injury.

In MDMA-induced hepatotoxicity, metabolism appears to play an important role and has been the subject of several studies (Tucker et al., 1994; Malpass et al., 1999; de la Torre et al., 2000; Maurer et al., 2000; Segura et al., 2001; Jones et al., 2005; Milhazes et al., 2006;
Akhter et al., 2008). MDMA is metabolized by two main routes: 1) $O$-demethylenation of the ring leading to 3,4-dihydroxymetamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA) followed by methylation by catechol-$O$-methyl-transferase (COMT) to 4-hydroxy-3-methoxyamphetamine (HMMA) or glucuronidation, or sulfation; and 2) chain conversion by $N$-dealkylation to 3,4-methylenedioxyamphetamine (MDA) followed by oxidation to benzoic acid derivates (Fig. 1).

Human hepatic MDMA $O$-demethylation and $N$-dealkylation are catalyzed by several cytochrome P450 (P450) enzymes. It has been suggested that CYP2D6 accounts for approximately 30% of MDMA metabolism, but involvement of other hepatic P450 enzymes, such as CYP1A2, CYP3A4, CYP2B6, and CYP2C19, has been reported (Meyer et al., 2008). However, the contribution of CYP3A4 and CYP1A2 to MDMA metabolism and what metabolites are being formed by these enzymes is less clear (Lin et al., 1992; Tucker et al., 1994; Kreth et al., 2000; Maurer et al., 2000). In particular, the reactive MDMA metabolites HHMA and HHA have been associated with a variety of toxic events, including hepatotoxicity (Carvalho et al., 2004a). Therefore, knowledge about the involvement of specific P450s in reactive metabolite formation is very important. Furthermore, genetic differences in P450 activity and/or expression might be responsible for the large differences in human susceptibility toward MDMA hepatotoxicity.

At present, several questions about the pharmacology of acute toxicity of MDMA remain unanswered. The pharmacokinetics of MDMA follows a nonlinear model, which results in a disproportional MDMA increase in plasma; with increasing MDMA intake, a relatively higher concentration of the parent MDMA is found in plasma compared with MDMA metabolites (de la Torre et al., 2000). This nonlinear kinetic model presented for MDMA at high dose levels has been attributed to autoinhibition or metabolic inactivation of CYP2D6 by the parent compound (de la Torre et al., 2000; Van et al., 2006).

In this study, we aimed for a better understanding of the role of MDMA metabolism in hepatotoxicity. MDMA metabolism was investigated in rat liver microsomes and in immortalized human liver epithelial cells (THLE) stably transfected with CYP1A2, CYP3A4, or CYP2D6. These P450s were selected because of their relative abundance in the human liver (Evans and Relling, 1999) and major contribution to MDMA metabolism. After transfected THLE cells were exposed to MDMA, P450 enzyme activity was measured and cytotoxicity was determined. In addition, metabolite formation was demonstrated using liquid chromatography/tandem mass spectrometry (LC-MS/MS) analyses. Furthermore, the effect of the phase II enzymes COMT and glutathione (GSH)/glutathione-S-transferase (GST) on MDMA-induced cytotoxicity in THLE cells was studied.

**Materials and Methods**

**Chemicals and Reagents.** DL-MDMA, DL-3,4-MDA, DL-HHMA, and DL-HMA were obtained from Duchefa-Farma (Haarlem, Netherlands). 6-naphthoflavone ($\beta$-NF), quinidine (QUI), troleandomycin (TAO), 2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone (RO 41-0960), buthionine sulfoximine (BSO), 3-(3,5-dimethylthia-
zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and methoxyresorufin (MTR) were purchased from Sigma-Aldrich (St. Louis, MO), 7-Benzoylxy-4-(trifluoromethyl)coumarin (BFC) and 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC) were obtained from BD Gentest (Woburn, MA).

**Rat Liver Microsome Fractions.** The liver was obtained from a healthy male rat and stored at −70°C. Tissue samples were weighed and homogenized in 10 ml of Tris-HCl buffer (50 mM Tris-HCl: 1.15% KCl, pH 7.4) per gram of tissue, using a Potter-Elvehjem Teflon-glass homogenizer (B. Braun Bio-technology International, Melsungen, Germany). Afterward, samples were centrifuged at 10,000 rpm for 25 min at 4°C. Subsequently, supernatants were pipetted into a clean ultracentrifuge tube and centrifuged for 1.25 h at 30,000 rpm at 4°C to separate the microsomal (pellet) from the cytosolic (supernatant) fractions. Pellets were resuspended in 1 ml of sucrose solution (0.25 M) per gram of original tissue. Aliquots of the microsomal fractions were stored at −70°C until used for analysis. Protein contents of isolated microsomes were calculated according to the method of Lowry et al. (1951) using bovine serum albumin as a protein standard.

**Cell Culture.** THLE cells, stably transfected with genes encoding for either CYP1A2, CYP3A4, or CYP2D6, were kindly provided by Nestec Ltd. (Lausanne, Switzerland). Cells were cultured on fibronectin/collagen-coated flasks in 1:1 Dulbecco’s modified Eagle medium/Ham’s F-12 nutrient mix (DMEM/F12, Invitrogen, Breda, Netherlands) supplemented with 0.33 mM retinoic acid (Sigma-Aldrich), 50 nM 3,3’,5-triiodo-l-thyronine sodium salt (Sigma-Aldrich), 5 mg/ml human epidermal growth factor (Sigma-Aldrich), 15 µg/ml bovine pituitary extract (Invitrogen), 0.2 µM hydrocortisone (Sigma-Aldrich), 500 nM 17β-estradiol (Sigma-Aldrich), 0.3% bovine serum albumin (Sigma-Aldrich), 1% insulin-transferrin-selenium (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% nonessential amino acids (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified atmosphere at 5% CO2.

**Phase 1 Enzyme Activity in Rat Liver Microsomes.** P450 activities were determined by incubating rat liver microsomes (34 µg/ml and 68 µg/ml for CYP3A2 and CYP2D1, respectively) with 0.5 M phosphate buffer (pH = 7.4), which contained a NADPH-regenerating system [20 mg/ml NADP (MTR) or 1 mg/ml NADP (CYP3A2), 20 mg/ml glucose-6-phosphate, 13.3 mg/ml MgCl2, and 40 U/ml glucose-6-phosphate dehydrogenase]. To measure CYP3A2 activity, microsomes were preincubated for 10 min at 37°C with α-NF and QUI. Microsomes used to study CYP2D1 activity were also preincubated with α-NF and TAO. Enzyme substrates were 50 µM BFC dissolved in acetonitrile (CYP3A2) or 1.5 µM AMMC dissolved in acetonitrile (CYP2D1). After 30-min incubation at 37°C, the reactions were stopped by adding 75 µl of 0.5 M Tris base (20%) in 80% acetonitrile. To measure CYP1A2 activity, we previously preincubcated microsomes for 10 min at 37°C with TAO and QUI, and we performed a methoxyresorufin-O-deethylation assay in which 50 µl of enzyme buffer mix [50 mM Tris-buffer/5 mM MgCl2 (pH = 7.8) with 17 µg/ml rat liver microsomes] was incubated for 10 min at 37°C. Then, 50 µl of substrate buffer mix was added, which contained 50 mM Tris-buffer/5 mM MgCl2 (pH = 7.8), 40 µM dicumarol, 4 µM TMR dissolved in dimethyl sulfoxide, and 3 mM NADPH. For CYP1A2, fluorescence was measured every 80 s for 26 min using a FLUOstar Galaxy (BMG Labtech). For CYP2D6, fluorescence was measured every 80 s for 26 min using a FLUOstar Galaxy (BMG Labtech). For CYP2D6, fluorescence was measured once at 80 s. The excitation/emission filters used to measure the activity of CYP1A2, CYP3A2, and CYP2D1 were 530/590, 410/510, and 405/460 nm, respectively.

**Phase 1 Enzyme Activity Assay in THLE Cells.** To determine phase I enzyme activity, 2.5 × 105 THLE cells were plated onto fibronectin/collagen-coated 24-well plates. The next day, cells were exposed to MDMA or its metabolites for 24 h. Fluorescence-based P450 assays were performed according to the methods of Donato et al. (2004) with minor modifications. Exposure medium was replaced, and 300 µl of incubation medium (DMEM/F12 supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin) was added to each well. Incubation medium contained 5 µM MTR (10 min), 50 µM BFC (2 h), or 100 µM AMMC (6 h) to determine CYP1A2, CYP3A4, or CYP2D6 activity, respectively (experimental conditions are shown in Table 1). For inhibition studies, specific enzyme inhibitors were added 2 h before the substrate and compounds were added (experimental conditions are shown in Table 2). The reactions were stopped by transferring the supernatants into a new plate. The cells were used to study the cytotoxicity levels after exposure. Glucuronidase/sulfatase (111000 units/ml and 1079 units/ml, respectively) was added to the supernatant and incubated for 2 h at 37°C. Finally, the reactions were quenched by diluting the samples (1:2) in 0.25 M Tris in 60% (v/v) acetonitrile. Activity levels of specific P450s were fluorometrically quantified using a FLUOstar Galaxy V4.30-0/Stacker Control V1.02-0 (BMG Labtechnologies), with the identical excitation/emission wavelength as described above.

**Cytotoxicity Assay.** The cell viability was determined by measuring the cells’ capability to reduce MTT to the blue-colored formazan after 1-h incubation. Quantification of the formazan dye was measured spectrophotometrically at 595 nm (FLUOstar Galaxy; BMG Labtechnologies). Metabolite exposure (HHMA) gave a brown-dark color formation in the medium; MTT results were corrected by these colored formations.

**LC-MS/MS.** For MDMA metabolite quantification, 250 µl of the incubation medium was treated with 10 µl glucuronidase/sulfatase (111000 units/ml and 1079 units/ml, respectively) for 2 h at 37°C; afterward, samples were treated with 250 µl of methanol and then centrifuged at 15,000 rpm for 10 min. Finally, 350 µl of supernatants were mixed with 350 µl of MilliQ water. Then, 10-µl aliquots were injected into a high-performance liquid chromatography (HPLC) system consisting of two HPLC pumps (PE200 series; Applied Biosystems, Foster City, CA), an autosampler (HTC-PAL, Zwingen, Switzerland), and an API4000 mass spectrometry (MS) detector equipped with an electrospray interface (Applied Biosystems). The HPLC column was a Luna C18 (150 × 2.0 mm; Phenomenex, Torrance, CA) that was eluted with mobile phase A, consisting of a mixture of 10 mM ammonium formate and 0.1% formic acid in water, and mobile phase B, consisting of acetonitrile. The flow rate was set at 200 µl/min using a linear gradient run as follows: 1 min, 90% A; 5 min, 70% A; 5 min, 70% A. The HPLC column was equilibrated with 90% A for 5 min before the next injection. The electrospray interface of the MS was operated at a voltage of 5000 V and a source temperature of 400°C. The entrance and declustering potentials were set at 10 V and 140 V, respectively. Tandem MS analysis was performed in positive multiple reaction monitoring (MRM) mode. The collision energy was set at 17 V. The transitions were set at m/z 194.2/163.2 (DMDA), m/z 180.2/163.1 (MDMA), m/z 182.2/151.1 (HHA), m/z 168.1/151.1 (HHA), m/z 168.1/151.1 (HHA).
Results

Effects of MDMA on Phase I Enzyme Activity. Rat liver microsomes. Rat liver microsomes were used to determine the inhibitory effect of MDMA on CYP1A1/2, CYP3A2, and CYP2D1 activities (Fig. 2). Exposure to MDMA (0–10 mM) for 30 min resulted in a concentration-dependent decrease of CYP1A1/2, CYP3A2, and CYP2D1 activity with IC50 values of 2124.0, 4.75, and 3.72 μM, respectively (Fig. 2). Specific P450 inhibitors α-NF, TAO, and QUI were used to calculate the relative inhibitory potency of MDMA for CYP1A1/2, CYP3A2, and CYP2D1, respectively. The specificity of the inhibitors was tested by determining the inhibitory potency of each inhibitor on CYP1A1/2, CYP3A2, and CYP2D1 activity (data not shown). To measure CYP1A1/1 activity, CYP3A2 and CYP2D1 were inhibited. CYP3A2 activity was determined after inhibition of CYP1A1/2 and CYP3A2. In the same way, CYP2D1 was studied with the inhibition of CYP1A1/2 and CYP3A2. The inhibitor concentrations used were chosen so that no inhibition of the target P450 was seen but full inhibition of the other P450 was obtained. In this way, MDMA inhibitory effects on specific rat P450 could be singled out.

The potency for CYP1A1/2 inhibition by MDMA was approximately 800-fold less than α-NF-inhibited CYP1A1/2 activity (Fig. 2A). α-NF displayed a dual effect in the CYP1A inhibition, which is very likely due to the presence of both CYP1A1 and CYP1A2, CYP3A2 activity was inhibited by TAO, which inhibited 50% of the CYP3A2 activity at 0.6 μM. The same concentration of MDMA decreased the CYP3A2 enzymatic activity by approximately 16%. MDMA was approximately 8-fold less potent in inhibiting CYP2D1 activity than TAO (Fig. 2B). MDMA and QUI were equipotent in inhibiting CYP2D1 activity with IC50 values of 3.7 and 2.4 μM, respectively (Fig. 2C). MDMA relative inhibitory potency of P450 was CYP2D1>CYP3A2>CYP1A1/2.

THLE cells. The THLEs transfected with single P450 were used as in vitro model to study the effect of MDMA exposure on specific P450 activity and the subsequent effect on liver cell viability. The THLE cells transfected with an empty vector (THLE-Neo) did not show any CYP1A2, CYP2D6, or CYP3A4 activity (data not shown). At the highest concentration tested (4 mM), a 24-h MDMA exposure resulted in a 76.0% inhibition of CYP1A2 activity and 100% inhibition of CYP3A4 and CYP2D6 activities in the THLE-1A2, THLE-3A4, and THLE-2D6 cell lines, respectively (Fig. 3).

The potency of P450 inhibition by MDMA in THLE cells was CYP2D6 (IC50 = 75.6 μM) ≫ CYP3A4 (IC50 = 233 μM) ≫ CYP1A2 (IC50 = 822 μM). Relative potencies of inhibition by MDMA were 16,182-, 20-, and 1850-fold less for CYP1A2, CYP3A4, and CYP2D6 compared with their corresponding P450-specific inhibitors.

Cytotoxicity by MDMA and Its Metabolites in THLE Cells. Effects of phase I metabolism. To some extent, the inhibition of P450 activities by MDMA was caused by cytotoxicity. The toxic potential of MDMA and the metabolites MDA, HHMA, and HMA was determined in the THLE-Neo cells transfected with an empty vector, and thus did not contain any P450 activity. Another reactive MDMA metabolite, HHMA, that can be formed through conversion of MDA or HMA (Fig. 1) was not included in our studies because of its low prevalence in human samples (de la Torre et al., 2004). All tested compounds induced cytotoxicity in a concentration-dependent manner after a 24-h exposure (Fig. 4). The phase I metabolite HHMA was most potent in inducing cytotoxicity with an apparent IC50 value of 473 μM. MDA (phase I metabolite) and HMA (phase II metabolite) showed approximately the same toxic potential as the parent compound (MDMA). The order of toxic potential was HHMA (IC50 = 473 μM) >> MDA (IC50 = 2.7 mM) > MDMA (IC50 = 3.8 mM) > HMA (IC50 = 4.6 mM). The maximal decrease of cell viability after...
24 h at 10 mM was 61.2 (MDMA), 69.6 (MDA), and 52.8% (HMA), respectively. Exposure of the THLE-Neo cells to HHMA caused a concentration-dependent brown-dark colored turbidity in the cells (data not shown) and a concomitant increase in cytotoxicity. At the highest concentration of HHMA tested (1 mM), cell viability decreased by 53.5% after 24 h.

To determine the role of specific P450 on MDMA metabolite formation and subsequent cytotoxicity, THLE-1A2, THLE-3A4, and THLE-2D6 were exposed to various concentrations of MDMA with and without specific P450 inhibitors. In all THLE cell lines, MDMA induced cytotoxicity in the millimolar range after 24-h exposure. At concentrations higher than 3 mM MDMA, a maximal 14.4% decrease in THLE-1A2 cell viability was observed (Fig. 5A). In THLE-2D6 cells, MDMA exposure decreased cell viability by 17.1 (2 mM), 29.8 (3 mM), and 25.3% (4 mM) compared with vehicle-treated control cells (Fig. 5C). In contrast, THLE-3A4 cell viability was significantly decreased (up to 61.9%) compared with control cells at the highest concentrations tested (Fig. 5B).

Cell viability was also determined in each cell line after coincubation of MDMA and the specific P450 inhibitors α-NF, TAO, or QUI at noncytotoxic concentrations (Table 2). Coincubation of THLE-CYP1A2 with α-NF did not alter cell viability compared with cells exposed to MDMA alone, suggesting a relatively low impact of CYP1A2-mediated metabolism of MDMA on its bioactivation (Fig. 5A). CYP3A4 inhibition by TAO increased MDMA-induced cytotoxicity, especially at the 1 mM concentration where CYP3A4 activity was not completely abolished (Fig. 3B). On the other hand, CYP2D6 inhibition by QUI showed a significant decrease in cytotoxicity compared with MDMA alone at 2 (13.9%) and 3 mM (15.2%) (Fig. 5C). This result confirms the important role of CYP2D6 in the bioactivation of MDMA.

Effects of phase II metabolism. The role of phase II metabolism in detoxification of MDMA phase I metabolites was determined in the THLE cell lines. The presence of GST in THLE cells was described previously (Cavin et al., 2001). Western blot analysis in our laboratory has confirmed the presence of COMT protein in the THLE cell line (data not shown).

THLE cells were coexposed with MDMA and specific phase II enzyme inhibitors for 24 h. Inhibition of COMT activity by Ro 41-0960 (10 μM) and GST activity by BSO (50 μM) did not result in a substantial change in MDMA-induced cytotoxicity in the THLE-1A2 cell line (Fig. 5D). A significant decrease of THLE-1A2 cell viability by 24.2% was observed upon GST inhibition, compared with MDMA alone, but only at the highest concentration of MDMA tested. In THLE-3A4 cells, a small, yet insignificant effect of COMT inhibition on MDMA-induced toxicity was observed. In contrast, GST inhibition further increased MDMA-induced cytotoxicity in the THLE-3A4 cells significantly at 2 (46.5%) and 3 mM (25.9%) compared with MDMA alone (Fig. 5E).

In THLE-2D6 cells, both COMT and GST inhibition significantly affected MDMA-induced cytotoxicity in a concentration-dependent manner (Fig. 5F). In MDMA-exposed THLE-2D6 cells, COMT inhibition significantly increased cytotoxicity of 35.2, 28.7, and 28.6% at 2, 3, and 4 mM MDMA, respectively, compared with MDMA exposure alone. GST inhibition only significantly decreased THLE-2D6 cell viability by 13.0% after MDMA exposure at 4 mM.
To confirm the formation of metabolites in each cell line, culture media of THLE-1A2, THLE-3A4, and THLE-2D6 cells exposed to 1 mM MDMA were analyzed by using LC-MS/MS (Fig. 6). The results show that MDMA metabolism actually occurs in the transfected THLE cell lines. Only two metabolites (MDA and HMMA) could be measured and quantified depending on the THLE cell line used. The THLE-1A2 cells showed the highest metabolic conversion of MDMA with MDA as the only metabolite. This effect indicates that CYP1A2 is the major P450 enzyme involved in formation of MDA. MDA formation was also observed by the THLE cells containing either CYP3A4 or CYP2D6, but the conversion rate was almost one order of magnitude less than that observed for THLE-CYP1A2 cells at 1.36e-03, 1.41e-03, and 8.79e-03 pmol/min, respectively. Furthermore, it was noticeable that the metabolite HMMA was only formed by THLE-2D6 cells at 1.88e-03 pmol/min, indicating a specific role of CYP2D6 in this case.

**Discussion**

The involvement of P450 and phase II enzymes in MDMA metabolism and their role in MDMA toxicity is not completely understood. In the stably transfected human liver cell line THLE with CYP1A2, CYP3A4, or CYP2D6, we have studied the role of these specific P450 enzymes on MDMA bioactivation. Then, metabolite formation was confirmed with LC-MS/MS analysis.

MDMA metabolism mainly takes place in the liver by CYP1A2, CYP3A4, and CYP2D6. Meyer et al. (2008) have shown that MDMA enantiomers differ in their affinity to these P450; nevertheless, we...
studied a racemic mixture of MDMA because MDMA consumers use a mixture. Most research focused on the role of CYP2D6 in MDMA toxicity. Several authors describe the influence of different allelic variants of CYP2D6 on MDMA-dependent toxic effects (Ramamorthy et al., 2002; Carmo et al., 2006). In contrast, de la Torre et al. (2005) suggested that nongenetic differences (e.g., autoinhibition) of CYP2D6 have a significant impact on MDMA pharmacokinetics and toxicity. Although CYP3A4 and CYP1A2 are known to play a role in MDMA metabolism, little attention has been focused on these P450 enzymes. This observation is remarkable in the case of CYP3A4, because approximately 30% of the human hepatic P450 content is CYP3A, whereas the relative abundance of CYP1A2 and CYP2D6 in the human liver is only 13 and 2%, respectively (Shimada et al., 1994).

MDMA seems to be responsible for the inhibition of its own detoxification process, as was previously described for CYP2D6, but our present study showed that this was also the case for CYP3A4 and CYP1A2 (Segura et al., 2005; Yang et al., 2006). The catalytic autoinhibition by MDMA was observed in rat liver microsomes and in THLE cells expressing individual human P450 enzymes. In both systems, the relative potency of catalytic inhibition by MDMA was CYP2D6 > CYP3A4 > CYP1A1. In theory, inhibition of CYP2D6 activity by MDMA could result in metabolic compensation by CYP1A2 and CYP3A4 in vivo (Lin et al., 1992; Kreth et al., 2000; Maurer et al., 2000; O’Mathuna et al., 2008). This result means that the role of CYP3A4 could become more important than CYP2D6 in MDMA metabolism at higher concentrations.

A divergent MDMA-induced cytotoxicity pattern was observed in the THLE cell lines. A wide range of MDMA concentrations was used, as has been described previously (Nakagawa et al., 2009), because MDMA levels in the liver can exceed the levels found in plasma.

MDMA induced the highest decrease in cell viability in THLE-3A4 cells, followed by the THLE-2D6 cells. THLE-1A2 cells showed only marginal cytotoxicity upon MDMA exposure. Our results are in contrast with a study by Carmo et al. (2006) who described that the CYP3A4 enzyme did not enhance MDMA toxicity in Chinese hamster lung fibroblast V79 cells transfected with individual P450 enzymes. This difference might indicate that cell-specific properties play an additional role in the cytotoxicity of MDMA or its metabolites. We further investigated the implications of P450 activity on MDMA toxicity by using concurrent exposure of the cells with specific P450 inhibitors. In all three cell lines, MDMA-induced cytotoxicity was affected the most by P450 inhibitors at MDMA concentrations where P450 activity was not completely inhibited. Apparently at lower MDMA concentrations (<1 mM), insufficient metabolites are being formed to cause cytotoxicity in our in vitro system. This finding was also described by Carvalho et al. (2002). At the highest MDMA concentration tested (4 mM), cytotoxicity was caused by MDMA itself, because phase I metabolism was completely inhibited by MDMA and no metabolites could be formed. Due to the high concentration of MDMA, this effect was considered to be a nonspecific cytotoxic effect. CYP2D6 inhibition by QUI resulted in an increase of THLE-2D6 cell viability after MDMA exposure, clearly indicating the significance of CYP2D6 in MDMA bioactivation. In contrast, blocking CYP1A2 and CYP3A4 enzymes did not counteract the MDMA-induced cytotoxicity. In THLE-3A4 cells, MDMA-induced toxicity was even weaker than CYP3A4 inhibition by QUI. Because TAO competes with MDMA for the CYP3A4 active site, this could potentially lead to a partial inhibition of CYP3A4 activity (Ma et al., 2000). Alternatively, the combination of TAO and CYP3A4-formed MDMA metabolites could generate extra stress to the cell, leading to enhanced cytotoxicity.

We also studied the implications of phase II enzymes COMT and GSH/GST in MDMA-mediated toxicity. Once again, the effects of phase II inhibition were most pronounced at MDMA concentrations where phase I metabolism was not completely inhibited by MDMA itself. COMT inhibition by Ro 41-0960 only had a minor effect on MDMA-induced cytotoxicity in THLE-1A2 and THLE-3A4 cells. In THLE-2D6, however, deactivation of COMT resulted in increased toxicity by MDMA. This activity demonstrates the relevance of COMT in the deactivation of reactive metabolites that arise from CYP2D6-mediated MDMA conversion. Other studies have also shown the influence of genetic variation in COMT activity on MDMA-induced toxicity (de la Torre et al., 2004; Schilt et al., 2009). Blocking GST activity by BSO caused a significant increase of MDMA-induced toxicity in all three THLE cell lines tested. Yet, this effect was most pronounced in THLE-3A4 cells, where coincubation with BSO increased MDMA-induced cytotoxicity by 46.6% compared with MDMA alone at 2 mM. Although MDMA metabolites have been shown to inhibit GST at higher concentrations (Carvalho et al., 2004b), these concentrations may not be reached in the THLE cells. Our data suggest a key role for GST in deactivating the reactive metabolites formed after CYP3A4-mediated MDMA conversion.

We propose that each specific P450 enzyme yields a different metabolic MDMA profile, which results in a different cytotoxic potential. In our experiments, the toxic potential of MDMA and its metabolites was shown to be HHMA > MDA > MDMA = HMA. As expected, the toxic potential increased upon phase I metabolism, whereas the presence of phase II metabolism had a reducing effect on cytotoxicity. HHMA and HHA are catechol metabolites that can generate ortho-quinones. These quinones are highly reactive molecules, which can undergo redox cycling and produce the formation of reactive oxygen species and reactive nitrogen species (Monks et al., 2004). As a consequence of alkylation and redox cycling, the o-quinones can generate adducts with crucial cellular proteins and/or DNA promoting cellular damage (Bolton et al., 2000; Milhazes et al., 2006). In THLE-Neo cells, significant cytotoxicity was observed after exposure to HHMA at low concentrations. Upon HHMA exposure, a dark brown/black turbidity emerged in a concentration-dependent manner (data not shown). This effect was previously described as melanin-type polymer formations in the medium as the oxidation progressed. These polymer formations appeared to be a later stage cytotoxic event, resulting from depleted GSH levels in the cell (Carvalho et al., 2004b).
To verify our in vitro findings of specific P450 activities and MDMA metabolites, media for each incubated THLE cell line were analyzed for MDMA metabolites by LC-MS/MS. Only MDA and HMMA could be quantified in our study, and they were used as representatives of the two major MDMA metabolic pathways (Fig. 1). As expected, CYP1A2 metabolism of MDMA yields MDA as major phase I metabolite. HMMA appeared specifically after CYP2D6-mediated MDMA conversion, illustrating the significant role of CYP2D6 in bioactivation. Although CYP3A4-mediated MDMA metabolism resulted in bioactivation in our in vitro studies, only some MDA but no other metabolite could be detected in the culture medium. COMT conversion after CYP3A4 metabolism of MDMA would theoretically yield HMMA, but this could not be detected. This outcome concurs with our findings that COMT plays a minor role in the detoxification of CYP3A4-formed MDMA metabolites. Most likely, the reactive phase I MDMA metabolites formed by CYP3A4 are rapidly inactivated by GSH conjugation. Conjugation of GSH with the reactive MDMA metabolites decreases the availability of the cellular GSH, which makes the cells more vulnerable to the effects of reactive compounds being formed, leading to harmful events.

The roles of phase I and phase II metabolism in MDMA toxicity have been described previously. Here, we show for the first time the different roles of specific enzymes. In our study, it was observed that CYP2D6, CYP3A4, and, to a lesser extent, CYP1A2 play an important role in MDMA bioactivation. COMT appears to be mainly involved in detoxification of CYP2D6-formed MDMA metabolites. GSH is important for inactivation of all MDMA metabolites, but its effect is most prominent on CYP3A4-formed MDMA metabolites. Considering the autoinhibition of CYP2D6 at relatively low MDMA concentrations and the relative abundance of CYP3A4 in the human liver, further studies are needed to elucidate the role of CYP3A4 and GSH/GST in MDMA bioactivation and detoxification. Elucidating the specific roles of these enzymes in MDMA toxicity is extremely important from a toxicological and clinical point of view. In some individuals, even a small amount of MDMA ingestion might lead to severe, potentially fatal intoxication, which is suggested to be attributed to variations in metabolizing and detoxifying enzyme activities. Further studies should confirm whether (genetic) differences in these metabolic enzymes can explain some of the interindividual differences in susceptibility toward MDMA toxicity.

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