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Differential roles of phase I and phase II enzymes in 3,4-methylenedioxymethamphetamine (MDMA)-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 cytochrome P450 2D6 (CYP2D6) enzyme in MDMA bioactivation, and less is known about the contribution of other CYP450 and phase II metabolism. In this study, the differential roles of phase I cytochrome CYP450 enzymes CYP1A2, CYP3A4 and CYP2D6 and phase II enzymes glutathione-S-transferase (GST) and catechol-O-methyltransferase (COMT) on the toxic potential of MDMA have been researched. MDMA acts as inhibitor of its own metabolism with a relative potency of inhibition of CYP2D>CYP3A>>CYP1A in rat liver microsomes and in human liver (THLE) cells transfected with individual CYP1A2, CYP3A4 or CYP2D6. Cytotoxicity measurements (by MTT) in THLE cells showed that the inhibition of phase I enzymes CYP1A2 by α-naphthoflavone and CYP3A4 by troleandomycin do not affect MDMA-induced cytotoxicity. MDMA metabolism by CYP2D6 significantly increased cytotoxicity, which was counteracted by CYP2D6 inhibition by quinidine. Inhibition of COMT by Ro-41-0960 and GST by BSO showed that COMT is mainly involved in detoxification of CYP2D6-formed MDMA metabolites, while GSH is mainly involved in detoxification of CYP3A4-formed MDMA metabolites. LC-MS/MS analyses of MDMA-metabolites in the THLE cell culture media confirmed formation of the specific MDMA metabolites and corroborated with the observed cytotoxicity. Our data suggest that not only CYP2D6 but also CYP3A4 plays 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.
Introduction:

MDMA (3,4-methylenedioxymethamphetamine, "ecstasy") is an illicit drug of abuse with stimulant and hallucinogenic properties. Chemically, it is a ring-substituted phenylamine derivate which became widely available in Europe at the beginning of the 1990s. The use of MDMA is mainly associated with nightclubs, dance music and some sub-cultures, 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; de la Torre, et al. 2004; Schifano 2004; Carmo, et al. 2006). Cases of hepatotoxicity due to MDMA ingestion have also been reported, yet it is expected to involve many more cases which are subclinical and thus remain undetected. Subacute hepatotoxicity can ultimately lead to severe liver damage upon repeated exposure and MDMA can be considered to be a cause of drug-induced liver injury (DILI).

In MDMA-induced hepatotoxicity, metabolism appears to play an important role and has been 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 metabolism involves 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-methoxymethamphetamine (HMMA) or glucuronidation, or sulfatation and 2) chain conversion by N-dealkylation to 3,4-methylendioxyamphetamine (MDA) followed by oxidation to benzoic acid derivates (Figure 1).

Human hepatic MDMA O-demethylenation and N-dealkylation is catalyzed by several cytochrome P450 (CYP) enzymes. CYP2D6 has been suggested to account for approximately 30% of MDMA metabolism, but involvement of other hepatic CYP450 enzymes such as CYP1A2, CYP3A4, CYP2B6 and CYP2C19 has been reported (Meyer, et al. 2008). Yet 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). Especially 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 involvement of specific CYP450s in reactive metabolite formation is very important. Furthermore, genetic differences in CYP 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. Pharmacokinetics of MDMA follows a non-linear 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 non-linear kinetic model presented for MDMA at high dose levels has been attributed to auto-inhibition 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 CYP450 were selected because of their major relative abundance in the human liver (Evans, et al.1999) and contribution in MDMA metabolism. After exposure of the transfected THLE cells to MDMA, CYP enzyme activity was measured and cytotoxicity was determined. In addition, metabolite formation was demonstrated using LC-MS/MS analyses. Furthermore, the effect of the phase II enzymes COMT and GSH/GST on MDMA-induced cytotoxicity in THLE cells was studied.
Materials and Methods:

Chemicals and reagents

D,L- Methylendioxymethamphetamine (MDMA), D,L-3,4-Methylendoxyamphetamine (MDA), D,L-3,4-Dihydroxymethamphetamine (HHMA), D,L-4-hydroxy-3-methoxy-amphetamine (HMA), were obtained from Duchefa-Farma (Haarlem, The Netherlands). Alpha-naphthoflavone (α-NF), Quinidine (QUI), Troleandomycin (TAO), 2′-Fluoro-3,4-dihydroxy-5-nitrobenzophenone (RO 41-0960), Buthionine sulfoximine (BSO), 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Methoxyresorufin (MTR) were purchased from Sigma (St. Louis, MO, USA), 7-Benzyloxy-4-(trifluoromethyl)coumarin (BFC), and 3-[2-(N,N-Diethyl-N-methylammonium)ethyl]-7-methoxy-4-methycoumarin (AMMC) were obtained from BD-Gentest.

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 TRIS-HCl buffer (TRIS-HCL 50 mm: 1.15% KCl, pH 7.4) per g tissue, using a Potter-Elvehjem Teflon-glass homogenizer. Afterwards, samples were centrifuged at 10,000 rpm for 25 min at 4°C. Subsequently, supernatants were pipetted into a clean ultra-centrifuge tube and centrifuged for 1:15h at 30,000 rpm at 4°C to separate the microsomal (pellet) from the cytosolic (supernatant) fractions. Pellets were resuspended in 1 ml sucrose solution (0.25 M) per g 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 using bovine serum albumine (BSA) as protein standard (Lowry, et al. 1951).

Cell culture

Immortalized human liver epithelial cell line (THLE) are cells, stably transfected with either CYP1A2, CYP3A4 or CYP2D6 genes, and were kindly provided by Nestec Ltd. (Lausanne, Switzerland). Cells were cultured on fibronectine/collagen-coated flasks in 1:1 Dulbecco’s modified Eagle medium/Ham’s
F-12 nutrient mix (DMEM/F12, Gibco 11039-021) supplemented with 0.33 nM retinoic acid (Sigma R2625), 50 nM 3,3',5-Triiodo-L-thyronine sodium salt (T3, Sigma T5516), 5 ng/ml human Epidermal Growth Factor (EGF, Sigma E9644), 15 μg/ml Bovine Pituitary Extract (BPE, Invitrogen 13028-014), 0.2 μM Hydrocortisone (Sigma H4881), 500 nM Ethanolamine (Sigma E0135), 0.3% Albumin from bovine serum (BSA, Sigma A7888), 1% Insulin-Transferrin-Selenium (ITS, Gibco 41400), 1% Sodium Pyruvate (Gibco 11360), 1% Non-essential amino acids (NEAA, Gibco 11140), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco 15140). Cells were cultured at 37°C in a humidified atmosphere at 5% CO₂.

Phase I enzyme activity in rat liver microsomes

CYP450 activities were determined by incubating rat liver microsomes (34 μg/well and 68 μg/well for CYP3A2 and CYP2D1, respectively) with 0.5 M phosphate buffer, pH=7.4, containing NADPH-regenerating system (20 mg/ml NADP (CYP3A2) or 1 mg/ml NADP (CYP2D1), 20 mg/ml Glucose 6-phosphate, 13.3 mg/ml MgCl₂ and 40 U/ml G6PDH). To measure CYP3A2 activity, microsomes were preincubated during 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 20% 0.5 M Tris-base in 80% acetonitrile. To measure CYP1A2 activity, previously we preincubated microsomes during 10 min at 37°C with TAO and QUI and we performed a methoxyresorufin-O-deethylase (MROD) assay in which 50 μl of enzyme buffer mix (50 mM Tris-buffer/ 5 mM MgCl₂ pH=7.8 with 17 μg/well rat liver microsomes) were incubated for 10 min. at 37°C. Then, 50 μl of substrate buffer mix was added containing 50 mM Tris-buffer/ 5 mM MgCl₂ pH=7.8, 40 μM Dicumarol, 4uM MTR dissolved in DMSO and 3 mM NADPH. Fluorescence was measured for CYP1A2 every 80 seconds for 26 minutes using a FLUOstar Galaxy (BMG Labtechnologies). For CYP3A2 and CYP2D1 fluorescence was measured once at 80 seconds. The excitation/emission filters used for measuring the activity of CYP1A2, CYP3A2 and CYP2D1 were 530/590, 410/510 and 405/460 nm, respectively.
Phase I enzyme activity assay in THLE cells

To determine phase I enzyme activity, 2.5x10^5 THLE cells were plated onto fibronectine/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 after Donato et al. with minor modifications (Donato, et al. 2004). Exposure medium was removed and 300 μl of incubation medium (DMEM/F12 supplemented with 1% NEAA, 1% Sodium Pyruvate and 100 U/ml penicillin and 100 μg/ml streptomycin) was added to each well. Incubation medium contained MTR (5 μM, 10 minutes), BFC (50 μM, 2 hours) or AMMC (100 μM, 6 hours) to determine CYP1A2, CYP3A4 or CYP2D6 activity, respectively (experimental conditions are shown in Table 1). For inhibition studies, specific enzyme inhibitors were added 2 hours 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. To the supernatant, glucuronidase/sulfatase (111000 units/ml and 1079 units/ml, respectively) was added and incubated for 2h 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 CYP450s were quantified fluorimetrically using a FLUOstar Galaxy V4.30-0/Stacker Control V1.02-0 (BMG Labtechnologies); with the identical ex/em wavelength as it has been described above.

Cytotoxicity assay

The cell viability was determined by measuring the cells capability to reduce MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 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 formation.
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 2h at 37 °C; afterwards samples were treated with 250 µl methanol and then centrifuged at 15000 rpm during 10 min. Finally, 350 µl of supernatants were mixed with 350 µl MilliQ water. Then, 10 µl aliquots were injected into an HPLC system consisting of two HPLC pumps (PE200 series, Applied Biosystems, Foster City, CA, USA), an autosampler (HTC-PAL, Zwingen, Switzerland) and an API4000 MS detector equipped with an electrospary interface (Applied Biosystems, Foster City, CA, USA). The HPLC column was a Luna C18 (150 x 2.0 mm, Phenomenex, Torrance, CA, USA), which was eluted with mobile phase A consisting of a mixture of 10mM ammonium formate and 0.1% formic acid in water and with mobile phase B consisting of acetonitrile. The flow rate was set at 200 µL/min using a linear gradient run as follows: 90% A for 1 min, then to 70% A in 5 min followed by 5 min with 70% A. The HPLC column was equilibrated with 90% A for 5 min prior to the next injection.

The electrospary interface of the MS was operated at a voltage of 5,000 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 multi-reaction-monitoring (MRM) mode. The collision energy was set at 17 V. The transitions were set at m/z 194.2/163.2 (MDMA), m/z 180.2/163.1 (MDA), m/z 182.2/151.1 (HHMA), m/z 168.1/151.1 (HHA), m/z 196.2/165.1 (HMMA) and m/z 182.2/165.1 (HMA). The LC-MS/MS instrument was controlled by Analyst software package (version 1.4.2, Applied Biosystems).

Data analysis

Results are presented as mean ± SEM. All experiments were performed at least in triplicate in each independent experiment. Details of the number of experiments are described in each legend. Graph representation was performed using Prism 4.0 (GraphPad Software, San Diego, CA). Statistical significance was calculated using SPSS 16.0 for Windows. Significance was accepted at P-value < 0.05.
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 (Figure 2). Exposure to MDMA (0-10mM) for 30 min. resulted in a concentration-dependent decrease of CYP1A1/2, CYP3A2 and CYP2D1 activity with IC50 values of 2124.0 µM, 4.75 µM and 3.72 µM, respectively (Figure 2). Specific CYP 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 activity CYP3A2 and CYP2D1 were inhibited. CYP3A2 activity was determined after inhibition of CYP1A1/2 and CYP2D1. In the same way, CYP2D1 was studied with the inhibition of CYP1A1/2 and CYP3A2. The inhibitor concentrations used were chosen so no inhibition of the target CYP was seen but full inhibition of the other CYP450 was obtained. In this way, MDMA inhibitory effects on specific rat CYP450 could be single out.

The potency for CYP1A1/2 inhibition by MDMA was approximately 800-fold less than α-NF inhibited CYP1A1/2 activity (Figure 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 about 16% the CYP3A2 enzymatic activity. MDMA was about 8-fold less potent in inhibiting CYP3A2 activity than TAO (Figure 2B). MDMA and QUI were equipotent in inhibiting CYP2D1 activity with IC50 values of 3.7 µM and 2.4 µM, respectively (Figure 2C). MDMA relative inhibitory potency CYP450 was CYP2D1>CYP3A2>>CYP1A1/2.
THLE cells

The human liver epithelial cells (THLE) transfected with single CYP450 were used as in vitro model to study the effect of MDMA exposure on specific CYP450 activity and 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 (Figure 3). The potency of CYP450 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 16182-, 20- and 1850-fold less for CYP1A2, CYP3A4 and CYP2D6 compared with their corresponding CYP specific inhibitors.

Cytotoxicity by MDMA and its metabolites in THLE cells

Effects of Phase I metabolism

To some extent, the inhibition of CYP450 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, thus not containing any CYP activity. Another reactive MDMA metabolite, HHA, that can be formed through conversion of MDA or HHMA (Figure 1) was not included in our studies because if its low prevalence in human samples (de la Torre, 2004). All tested compounds induced cytotoxicity in a concentration-dependent manner after a 24-h exposure (Figure 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 maximum decrease of cell viability after 24-hours 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 HHMA tested (1 mM) cell viability was decreased with 53.5% after 24 hours.

To determine the role of specific CYP450 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 CYP inhibitors. In all THLE cell lines, MDMA induced cytotoxicity in the mM range after 24 hours of exposure. At concentrations higher that 3 mM MDMA, a maximum of 14.4% decrease in THLE-1A2 cell viability was observed (Figure 5A). In THLE-2D6 cells, MDMA exposure resulted in a decrease in cell viability with 17.1% (2 mM), 29.8% (3 mM) and 25.3% (4 mM) compared with vehicle-treated control cells (Figure 5C). Conversely, THLE-3A4 cell viability was significantly decreased up to 61.9% compared with control cells at the highest concentrations tested (Figure 5B).

Cell viability was also determined in each cell line after co-incubation of MDMA and the specific CYP inhibitors α-NF, TAO or QUI at non-cytotoxic concentrations (Table 2). Co-incubation of THLE-CYP1A2 with α-NF did not alter cell viability compared to cells exposed to MDMA alone, suggesting a relatively low impact of CYP1A2-mediated metabolism of MDMA on its bioactivation (Figure 5A). CYP3A4 inhibition by TAO increased MDMA-induced cytotoxicity, especially at the 1 mM concentration where CYP3A4 activity was not completely abolished (Figure 3C). On the other hand, CYP2D6 inhibition by QUI showed a significant decrease in cytotoxicity compared with MDMA alone at 2 mM (13.9%) and 3 mM (15.2%) (Figure 5C). This 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 Glutathione S-Transferase (GST) in THLE cells was described previously (Cavin et al. 2001). Western blot analysis in our lab has confirmed the presence of Catechol-O-Methyl Transferase (COMT) protein in the THLE cell line (data not shown).

THLE cells were co-exposed 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 (Figure 5D). Only at the highest
concentration of MDMA tested, a significant decrease of THLE-1A2 cell viability by 24.2% was observed upon GST inhibition, compared to MDMA alone. In THLE-3A4 cells, a small, yet not significant effect of COMT inhibition on MDMA-induced toxicity was observed. Conversely, GST inhibition further increased MDMA-induced cytotoxicity in the THLE-3A4 cells significantly at 2 mM (46.5%) and 3 mM (25.9%) compared with MDMA alone (Figure 5E).

In THLE-2D6 cells, both COMT and GST inhibition significantly affected MDMA-induced cytotoxicity in a concentration-dependent manner (Figure 5F). In MDMA-exposed THLE-2D6 cells, COMT inhibition resulted in a significant increase of cytotoxicity of 35.2%, 28.7% and 28.6% at 2 mM, 3 mM and 4 mM MDMA, respectively, compared to MDMA-exposure alone. GST inhibition only significantly decreased THLE-2D6 cell viability with 13.0% after MDMA exposure at 4mM.

**LC-MS/MS**

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 analysed using LC-MS/MS (Figure 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 only metabolite. This 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 and CYP2D6, but conversion rate was almost one order of magnitude less as observed for THLE-CYP1A2, was respectively 1.36e-03, 1.41e-03 and 8.79e-03 pmol/min. 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 CYP450 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 CYP450 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. have shown MDMA enantiomers differ on their affinity to these CYP450 (Meyer et al. 2008); nevertheless, we studied a racemic mixture of MDMA since 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 (Ramamoorthy, et al. 2002; Carmo, et al. 2006). Conversely, de la Torre and co-workers suggested non-genetic differences, e.g. auto-inhibition, of CYP2D6 to have a significant impact on MDMA pharmacokinetics and toxicity (de la Torre, et al. 2005). While CYP3A4 and CYP1A2 are known to play a role in MDMA metabolism, little attention has been focused on these CYP450 enzymes. This is remarkable in the case of CYP3A4, since about 30% of the human hepatic CYP450 content is CYP3A, whereas the relative abundance of CYP1A2 and CYP2D6 in the human liver are only 13% and 2%, respectively (Shimada, et al. 1994).

MDMA seems responsible for the inhibition of its own detoxification process, as was previously described for CYP2D6, but our present study showed this also to be the case for CYP3A4 and CYP1A2 (Segura, et al. 2005; Yang, et al. 2006). The catalytic auto-inhibition by MDMA was observed in rat liver microsomes and in THLE cells expressing individual human CYP450 enzymes. In both systems, the relative potency of catalytic inhibition by MDMA was CYP2D>CYP3A>>CYP1A. Theoretically, 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 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 previously described (Nakagawa, et al. 2009), since 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. who described that the CYP3A4 enzyme did not enhance MDMA toxicity in Chinese hamster lung fibroblast V79 cells transfected with individual CYP450 enzymes (Carmo et al. 2006). 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 CYP450 activity on MDMA toxicity by using concurrent exposure of the cells with specific CYP inhibitors. In all three cell lines, MDMA-induced cytotoxicity was affected most by CYP inhibitors at MDMA concentrations where CYP 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 was also described by Carvalho et al (Carvalho et al 2002). At the highest MDMA concentration tested (4 mM), cytotoxicity was caused by MDMA itself, since phase I metabolism was completely inhibited by MDMA and no metabolites could be formed. Due to the high concentration of MDMA, this was considered 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 aggravated upon CYP3A4 inhibition by TAO. Since 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 on the other hand, deactivation of COMT resulted in increased toxicity by MDMA. This
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 co-incubation with BSO increased MDMA-induced cytotoxicity with 46.6% compared to MDMA alone at 2 mM. Although MDMA-metabolites have been shown to inhibit GST at higher concentrations (Carvalho et al. 2004b), these concentrations are most likely not 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 CYP450 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 while 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 (ROS) and reactive nitrogen species (RNS) (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). Previously, this effect was described as melanin-type polymers formation in the medium as the oxidation progressed. These polymers formation 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 CYP activities and MDMA metabolites, media of each incubated THLE cell lines were analyzed for MDMA metabolites by LC-MS/MS. Only MDA and HMMA could be quantified in our study, and used as representatives of the two major MDMA metabolic pathways (Figure 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 and no other metabolites 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 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. However, we show here for the first time the differential roles of specific enzymes. In our study, it was observed that not only CYP2D6, but also 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 auto-inhibition 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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References:


Human pharmacology of MDMA: pharmacokinetics, metabolism, and disposition. Ther Drug Monit 26(2): 137-44.


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Legends for figures:

**Figure 1.** MDMA, main metabolic pathway [1]. MDMA (3,4-methylendioxymethamphetamine), MDA (3,4-methylendioxyamphetamine), HHMA (3,4-dihydroxymetamphetamine), HHA (3,4-dihydroxyamphetamine), HMMA (4-hydroxy-3-methoxymethamphetamine), HMA (4-hydroxy-3-methoxyamphetamine).

**Figure 2.** CYP1A1/2, CYP3A2 and CYP2D1 activity in rat liver microsomes after a 30-min exposure to MDMA. (A, B, C) Inhibition of CYP450 activity by MDMA compared with specific inhibitors for CYP450 enzymes. Alpha-Naphthoflavone (α-NF), Troleandomycin (TAO) and Quinidine (QUI) were used as inhibitors for CYP1A1/2, CYP3A2 and CYP2D1 activity, respectively. Three independent experiments were performed and each concentration was tested in triplicate (N=9). Data are represented as a mean ± SEM of all experiments.

**Figure 3.** CYP1A2, CYP3A4 and CYP2D6 activity in transfected human liver epithelial (THLE) cells after a 24-h exposure to MDMA or the specific inhibitor α-NF (CYP1A2), TAO (CYP3A4) or QUI (CYP2D6). Data are represented as a mean ± SEM of three independent experiments, in which each concentration was tested in duplicate (N=6).

**Figure 4.** Cytotoxicity of MDMA and MDMA metabolites (MDA, HHMA, and HMA), tested in transfected human liver epithelial cell line with an empty vector (THLE-Neo). Cells were exposed for 24 hours and toxicity was measured as MTT. Data are represented as a mean ± SEM of two independent experiments and each concentration was tested in duplicate (N=4).

**Figure 5.** Cell viability in transfected human liver epithelial cells (THLE cells) expressing single CYP1A2, CYP3A4 or CYP2D6 after a 24-h incubation to MDMA. (A, B, C) MDMA-induced toxicity when phase I enzymes are inhibited (α-NF, TAO and QUI). (D, E, F) MDMA-induced toxicity when phase II enzymes are inhibited (Ro 41-0960, BSO). Data are represented as mean ± SEM of three independent experiments, where each concentration was tested in duplicate (N=6). (*) Significantly different from vehicle-treated control cells (P< 0.05). (†) Significantly different from cells treated with the same MDMA concentration alone (P< 0.05).
Figure 6. Detection MDMA-metabolite formation obtained by LC-MS/MS analyses. Media from THLE cells expressing single CYP1A2, CYP3A4 or CYP2D6 after a 24-h exposure to 1mM MDMA were analysed. Data are represented as mean ± SEM of two independent experiments, where each concentration was tested in triplicate (N=6). ND, not detected. (**) Significantly different metabolite formation after exposure to 1 mM MDMA (P< 0.01).
Table 1. Experimental conditions of fluorescence bioassay.

<table>
<thead>
<tr>
<th>CYP450</th>
<th>Substrate</th>
<th>Incubation time (min)</th>
<th>Concentrations (µM)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Microsomes</td>
<td>THLE cells</td>
</tr>
<tr>
<td>1A2</td>
<td>MTP</td>
<td>30</td>
<td>0-10</td>
</tr>
<tr>
<td>3A4</td>
<td>BFC</td>
<td>30</td>
<td>120</td>
</tr>
<tr>
<td>2D6</td>
<td>AMMC</td>
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<td>360</td>
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Table 2. Inhibitory conditions of fluorescence bioassay.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>Microsomes</th>
<th>THLE cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>α-Naphthoflavone (α-NF)</td>
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<td>CYP3A4</td>
<td>Troleandomycin (TAO)</td>
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<td>50</td>
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<tr>
<td>CYP2D6</td>
<td>Quinidine (Q)</td>
<td>20</td>
<td>10</td>
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<tr>
<td>COMT</td>
<td>Ro 41-0960</td>
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</tr>
<tr>
<td>GST</td>
<td>Buthionine sulfoximine (BSO)</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

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Figure 4

MDMA-metabolite toxicity study in THLE-Neo cells
Figure 6

THLE cell lines

Metabolite formation (pmol/min)

CYP1A2  CYP3A4  CYP2D6

ND  ND  ND

MDA  HMMA