Short Communication

IDENTIFICATION OF HUMAN CYTOCHROME P450 2D6 AS MAJOR ENZYME INVOLVED IN THE O-DEMETHYLATION OF THE DESIGNER DRUG P-METHOXYMETHAMPHETAMINE

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ABSTRACT:

p-Methoxymethamphetamine (PMMA) is a new designer drug, listed in many countries as a controlled substance. Several fatalities have been attributed to the abuse of this designer drug. Previous in vivo studies using Wistar rats had shown that PMMA was metabolized mainly by O-demethylation. The aim of the study presented here was to identify the human hepatic cytochrome P450 (P450) enzymes involved in the biotransformation of PMMA to p-hydroxymethamphetamine. Baculovirus-infected insect cell microsomes (P450) enzymes involved in the biotransformation of PMMA to p-hydroxymethamphetamine. Baculovirus-infected insect cell microsomes, pooled human liver microsomes (pHLMs), and CYP2D6 poor-metabolizer genotype human liver microsomes (PM HLMs) were used for this purpose. Only CYP2D6 catalyzed O-demethylation. The apparent K_m and V_max values in baculovirus-infected insect cell microsomes were 4.6 ± 1.0 μM and 92.0 ± 3.7 pmol/min/pmol P450, respectively, and 42.0 ± 4.0 μM and 412.5 ± 10.8 pmol/min/mg protein in pHLMs. Inhibition studies with 1 μM quinidine showed significant inhibition of the metabolite formation (67.2 ± 0.6%; p < 0.0001), and comparison of the metabolite formation between pHLMs and PM HLMs revealed significantly lower metabolite formation in the incubations with PM HLMs (67.3 ± 1.1%; p < 0.0001). According to these studies, CYP2D6 is the major P450 involved in O-demethylation of PMMA.

p-Methoxymethamphetamine [PMMA]; International Union of Pure and Applied Chemistry, IUPAC: [1-(4-methoxyphenyl)propane-2-yl](methyl)azane] is a new designer drug, listed as a controlled substance in many countries (European Communities, 2002). PMMA and its N-demethylated analog para-methoxymethamphetamine [IUPAC: 1-(4-methoxyphenyl)propane-2-ylazane] have been made responsible for a large number of fatalities all over the world (Felgate et al., 1998; James and Dinan, 1998; Bach et al., 1999; Kramer et al., 2001; Ling et al., 2001; Martin, 2001; Johansen et al., 2003). PMMA shares considerable pharmacological similarities with methylenedioxymethamphetamine (MDMA), but it lacks the amphetamine-like stimulant component of MDMA. It produced stimulant effects similar to N-methylbenzodioxolylbutanamine [IUPAC: 1-(1,3-benzodioxol-5-yl)butan-2-yl](methyl)azane] and dimethoxymethamphetamine (1-(3,4-dimethoxyphenyl)-2-aminopropane; IUPAC: 1-(2,5-dimethoxyphenyl)propane-2-ylazane) (Glenon et al., 1988, 1997; Glenon and Higgs, 1992; Young et al., 1999; Rangisetty et al., 2001). The only data available on the effects in humans have been published by Shulgin (1991), who described its effects as somewhat different from those of MDMA. Steel et al. (1992) showed long-term (possibly neurotoxic) effects on brain serotonin neurons for PMMA, although less potent compared with neurotoxic effects of MDMA.

Previous in vivo studies in the rat have shown that PMMA is metabolized mainly by O-demethylation to para-hydroxymethamphetamine (HO-MA) (Fig. 1) (Staack et al., 2003). The aim of the current study was to identify the human hepatic cytochrome P450 (P450) enzymes involved in the O-demethylation, to determine the kinetic constants for this metabolic reaction, and to compare the metabolite formation in pooled human liver microsomes (pHLMs) and in single donor human liver microsomes with CYP2D6 poor-metabolizer genotype (PM HLMs).

Materials and Methods

Materials. PMMA was obtained from Lipomed (Bad Säckingen, Germany); HO-MA was obtained from Sigma Chemie (Diesenhofen, Germany); and HO-AM was provided by Professor K. Pfleger (Homburg, Germany). NADP* was obtained from Biomol (Hamburg, Germany); isocitrate and isocitrate dehydrogenase were purchased from Sigma (Taufkirchen, Germany); and all other chemicals and reagents were obtained from Merck Biosciences (Darmstadt, Germany). The following microsomes were from BD Gentest (Woburn, MA) and delivered by NatuTec (Frankfurt am Main, Germany): baculovirus-infected insect cell microsomes containing 1 nmol/ml CYP2D6 (Supersomes), wild-type baculovirus-infected insect cell microsomes (control Supersomes), pHLMs (20 mg of microsomal protein/ml, 400 pmol total P450/mg protein), and PM HLMs (20 mg of microsomal protein/ml). After delivery, the microsomes were thawed at 37°C, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C until use. The activities of the control markers were as follows: 58 pmol product/(min × pmol P450) for CYP 2D6 Super-
somewhat (bufuralol 1′-hydroxylase activity) and 69 pmol/(mg × min) CYP 2D6 activity in pHLMs (bufuralol 1′-hydroxylase activity).

**Microsomal Incubations.** Incubation mixtures (final volume, 50 µl) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM MgCl₂, 5 mM isocitrate, 1.2 mM NADP⁺, 0.5 U/ml isocitrate dehydrogenase, 200 U/ml superoxide dismutase, and substrate at 37°C. The substrate was added after dilution of a 250 mM methanolic stock solution in buffer. The methanol concentration did not exceed 0.4% in any of the samples. Reactions were started by addition of the ice-cold microsomes and terminated with 5 µl of 60% (w/v) HClO₄. After addition of 1 µl of 0.5 mM HO-AM as internal standard (IS), the samples were centrifuged, and the supernatants were transferred to autosampler vials.

**Initial Screening Studies.** To investigate the involvement of particular P450 enzymes in PMMA metabolism, 100 µM PMMA and 50 pmol/ml CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, or CYP3A4 were incubated for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM or 90 mM Tris buffer, respectively.

**Kinetic Studies.** Duration of and protein content for all incubations were in the linear range of metabolite formation (data not shown). Kinetic constants were derived from incubations with PMMA concentrations between 2.5 and 400 µM (n = 2, each).

pHLM and cDNA-expressed P450 protein contents were 0.8 mg/ml and 20 pmol P450/ml, respectively. Incubation times were 25 and 2 min for pHLMs and cDNA-expressed P450, respectively. Less than 20% of substrate was metabolized in all incubations. Kinetic profiles and values were first estimated by visual inspection of Eadie-Hofstee plots, since deviations from simple Michaelis-Menten kinetics could be readily seen (Clarke, 1998). Apparent inhibitor Michaelis-Menten kinetics could be then estimated using GraphPad Prism 3.02 software (GraphPad Software Inc., San Diego, CA).

**Chemical Inhibition Studies.** The effect of 1 µM quinidine on HO-MA formation was assessed in incubations containing 0.8 mg of either pHLM or PM HLM. The Michaelis-Menten and Eadie-Hofstee plots (Fig. 1) were used to determine the metabolite concentration in the incubation mixtures, and the rate of metabolism was measured by a one-tailed unpaired t test.

**Comparative Studies between pHLMs and PM HLMs.** Incubations were carried out at 40 µM PMMA for 25 min using 0.8 mg of either pHLM or PM HLM protein/ml. Significance of differences in metabolite formation was compared with incubations without the inhibitor. The concentration of the inhibitor was based on average literature data (Clarke 1998). The inhibition experiments were performed with 40 µM PMMA, a substrate concentration that corresponds to the calculated Kᵢ value in pHLM. HO-MA formation was significantly inhibited by 67.2 ± 0.6% (p < 0.0001), which was further evidence of the involvement of CYP2D6 in the monitored reaction.

**Chemical Inhibition Studies.** To underline the importance of CYP2D6 in PMMA metabolism, the CYP2D6 specific inhibitor quinidine (1 µM) was added to incubation mixtures, and the rate of metabolism was compared with incubations without the inhibitor. The concentration of the inhibitor was based on average literature data (Clarke 1998). The inhibition experiments were performed with 40 µM PMMA, a substrate concentration that corresponds to the calculated Kᵢ value in pHLM. HO-MA formation was significantly inhibited by 67.2 ± 0.6% (p < 0.0001), which was further evidence of the involvement of CYP2D6 in the monitored reaction.

**Results and Discussion**

**LC-MS Procedure.** The applied LC-MS conditions provided baseline separation of HO-AM, HO-MA, and PMMA. The resulting calibration curves were linear over the tested calibration range (r² > 0.993) and were used to determine the metabolite concentration in the samples.

**Initial Screening Studies.** Among the nine P450 enzymes tested for possible HO-MA formation from PMMA, CYP2D6 was the only P450 capable of catalyzing this reaction.

**Kinetic Studies.** All incubations were carried out at initial rate conditions, a prerequisite for Michaelis-Menten kinetics. CYP2D6 showed a typical hyperbolic metabolism formation profile (Fig. 2A). The apparent Km value for CYP2D6 was determined to be 4.6 ± 1.0 µM, and Vmax was calculated to be 92.0 ± 3.7 pmol/min/pmol P450. The kinetic parameter for incubations using pHLMs was determined to be: apparent Km, 42.0 ± 4.0 µM and Vmax, 412.5 ± 10.8 pmol/min/mg protein. The Michaelis-Menten and Eadie-Hofstee plots (Fig. 2B and C) for the incubations with pHLMs did not indicate biphasic kinetics, which is consistent with the finding of the initial screening studies that only one P450 was involved in the O-demethylation of PMMA. Apparent Km values for CYP2D6 and for pHLM differed somewhat from one another. This may be due to the fact that non-specific protein binding was greater in pHLMs (0.8 mg/ml) compared with CYP2D6 microsomes (0.3 mg/ml). Such nonspecific binding to phospholipid membranes has been described for lipophilic and cat- ionic compounds at pH 7.4, which should be the case for PMMA (Margolis and Obach, 2003).

**Chemical Inhibition Studies.** To underline the importance of CYP2D6 in PMMA metabolism, the CYP2D6 specific inhibitor quinidine (1 µM) was added to incubation mixtures, and the rate of metabolism was compared with incubations without the inhibitor. The concentration of the inhibitor was based on average literature data (Clarke 1998). The inhibition experiments were performed with 40 µM PMMA, a substrate concentration that corresponds to the calculated Kᵢ value in pHLM. HO-MA formation was significantly inhibited by 67.2 ± 0.6% (p < 0.0001), which was further evidence of the involvement of CYP2D6 in the monitored reaction.

**Comparative Studies between pHLMs and PM HLMs.** To point out the importance of CYP2D6 in PMMA O-demethylation and to demonstrate differences in CYP2D6 poor metabolizer (PM) and CYP2D6 extensive metabolizer (EM), the metabolite formation rate of pHLM was compared with that of PM HLM. The metabolite formation rate in PM HLMs was significantly lower (87.3 ± 1.1%; p < 0.0001) than that of pHLMs. This remarkable difference in metabolism formation between PM and EM is further proof of the involvement of CYP2D6 in the major metabolic step of PMMA. The
CYP2D6 is the major P450 involved in O-demethylation of PMMA, and significant differences between PMs and EMs could be shown in vitro with PMs exhibiting lower metabolic clearances. PMMA might also be susceptible to drug-drug interactions with potent CYP2D6 inhibitory drugs leading to a decreased clearance of PMMA and elevated plasma concentrations. Of course, it cannot be concluded that these genetic polymorphisms and drug interactions are actually of clinical relevance. This would require in vivo studies in humans. Furthermore, it would be of particular interest to study whether a correlation between the reported fatalities and their CYP2D6 genotype exists.

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References


FIG. 2. Michaelis-Menten plots (A and B) and Eadie-Hofstee plot (C) for CYP2D6 (A), and pHLM (B and C).

Data points in A and B represent means (points) and ranges (error bars) of two individual measurements. Solid curves in A and B were calculated by nonlinear regression according to the Michaelis-Menten equation (one-site binding model).

The study design used has already successfully been applied for investigations on the metabolism of other designer drugs (Springer et al., 2003a,b).

The results of the current study are in accordance with the results of studies on the involvement of CYP2D6 in the O-demethylation of other methoxy-substituted amphetamine derivatives (Coutts et al., 1994; Wu et al., 1997; Bach et al., 1999). Our studies showed that